ENDOGENOUS SYNTHESIS OF GLYCINE

FROM HYDROXYPROLINE IN NEONATAL PIGS

A Dissertation

by

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ABSTRACT

This study was conducted to test the hypothesis that hydroxyproline is a novel and major substrate for endogenous synthesis of glycine in sow-reared pigs. At 0, 7, 14, and 21 days of age, neonatal piglets with a normal or low birth weight (BW) were sacrificed, and their tissue samples were obtained for metabolic studies, activities of glycine-synthetic enzymes, mRNA expression, and the localization of proteins for those enzymes. Moreover, normal and IUGR piglets received oral administration of glycine (0.2, 0.4, and 0.8 g/kg BW) between days 0 and 14 to evaluate a role for endogenous synthesis of glycine in the growth of piglets.

Results from the studies of normal birth-weight piglets demonstrated that the activities of hydroxyproline oxidase (OH-POX), proline oxidase (POX), alanine:glyoxylate transaminase (AGT), and 4-hydroxy-2-oxoglutarate aldolase (HOA), key enzymes for glycine synthesis from hydroxyproline, decreased in the liver and kidneys between postnatal day 0 and day 21, but increased in the pancreas and small intestine over the same period of time ($P < 0.05$). Similar results were obtained for expression of mRNAs for those enzymes. The enzymatic activities and expression of mRNAs for serine hydroxymethyl transferase (SHMT) and threonine dehydrogenase (TDH) increased between days 0 and 21 of age in most tissues ($P < 0.05$). Localization of OH-POX and POX shifted from periportal to perivenous hepatocytes in the liver as the animal grew. Tissues incubated with 0 to 5 mM hydroxyproline synthesized glycine in a concentration-dependent manner, but the conversion of glycine into serine was limited. The activities of OH-POX and

SHMT were lower in tissues of IUGR piglets ($P < 0.05$), compared with normal pigs. The activity of TDH did not differ between IUGR and normal piglets. Similar results were obtained for expression of mRNAs of those enzymes.

Oral administration of glycine to sow-reared IUGR and normal birth-weight piglets enhanced the concentrations of glycine and serine in plasma, as well as weight gain ($P < 0.05$), with the dose of 0.4 g glycine/kg BW being the most effective. Consistent with its growth-promoting effect, glycine supplementation augmented the phosphorylation of mechanistic target of rapamycin (MTOR), eukaryotic initiation factor 4E binding protein 1 (4E-BP1) and p70S6 kinase (p70S6K) in skeletal muscle, compared with control piglets.

The results of this research indicate that: (1) hydroxyproline, an abundant metabolite in sow's milk and a product of collagen degradation, is a more important substrate for glycine synthesis in tissues of young pigs than serine and threonine; (2) endogenous synthesis of glycine is insufficient for maximal growth of sow-reared IUGR and normal birth-weight piglets; and (3) glycine supplementation enhanced activity of the MTOR signaling pathway in skeletal muscle and growth performance of newborn piglets, especially those with a low birth weight. Collectively, these results indicate an important role for glycine in improving growth of and protein accretion in neonatal pigs.

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NOMENCLATURE

4E-BP1	4E binding protein 1
AA	Amino acid
AGT	Alanine:glyoxylate transaminase
BW	Body weight
EAA	Essential amino acid
Gly	Glycine
HOA	4-hydroxy-2-oxoglutarate aldolase
Hyp	Hydroxyproline
IUGR	Intrauterine growth restriction
meTHF	N^5-N^{10}-methylene-tetrahydrofolate
MTOR	Mechanistic target of rapamycin
NEAA	Non-essential amino acid
OH-POX	Hydroxyproline oxidase
P70S6K	Ribosomal protein S6 kinase
POX	Proline oxidase
SHMT	Serine hydroxymethyl transferase
TDH	Threonine dehydrogenase
THF	Tetrahydrofolate

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Introduction

As important compounds in humans and animals, amino acids serve numerous physiological functions (Wu et al. 2014). Only 20 of amino acids are building blocks for more than 700 cellular proteins. These 20 proteinogenic amino acids are: alanine, arginine, asparagine, aspartate, cysteine, glutamate, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, tyrosine, threonine, tryptophan, and valine. They have different side chains and chemical properties (Broer and Broer 2017; Wu 2013b). Other amino acids also participate in vital physiological functions including detoxification of ammonia through the urea cycle, and synthesis of polyamines and nitric oxide. Thus, exploring the frontier of knowledge of amino acid biochemistry and nutrition is critical for improving human and animal health.

Traditionally, based on nitrogen balance or growth of animals, nutritionists classified amino acids as nutritionally essential amino acids (EAAs) or non-essential amino acids (NEAAs). There is a rich history of studies on amino acid requirements in humans and other animals (Chung and Baker 1992; Kim et al. 2001; Millward 1997; Rose 1968; Simoni et al. 2002; Wu 2014). In the 1910s, T.B. Osborne and L.B. Mendel defined EAAs as "amino acids which cannot be manufactured *de novo* in the animal organism" (Osborne et al. 1917). Until the 1930s, based on results from a series of human and animal

experiments, W.C. Rose classified amino acids as EAAs or NEAAs based on the health status and body weight changes of human subjects consuming semi-purified diets lacking a specific amino acid (Rose 1968). In the late 1990s, D.H. Baker included the different ratios of amino acids to lysine (the ideal protein) in formulating diets for swine (Chung and Baker 1992; Kim et al. 2001). Neither NRC Swine Nutrition Requirements in 1998 nor D.H. Baker recognized the nutritional requirement for NEAAs (Kim et al. 2001). Growing evidence shows that arginine, cysteine, glycine, proline, and tyrosine, previously categorized as NEAAs, are required by many species since the endogenous synthesis of those AAs cannot meet the needs for the maintenance, optimum growth, and health of animals (Kim and Wu 2004; Wang et al. 2014a; Wang et al. 2013a; Wu 2013c; Wu et al. 2011; Wu et al. 2014; Wu et al. 2008; Wu et al. 1994). Recently, Wu (2013c) defined EAAs as amino acids whose carbon skeletons cannot be synthesized *de novo* or synthesized in amounts that are insufficient to meet the needs of animals for maintenance, growth, development, and health. Clearly, EAAs must be provided in diets to meet the nutritional requirements of mammals, birds, and fish (Wu 2013c). Nutritionally non-essential amino acids were previously defined as amino acids which can be synthesized in an adequate amount to meet the needs of maintenance, optimum growth, and health of animals. However, requirements of NEAAs are affected by many factors, including species, age, physiological status, and health conditions. Amino acids which are insufficiently synthesized at a specific physiological stage or under certain environmental conditions are classified as conditionally essential amino acids (CEAA). Those include glycine in poultry and milk-fed piglets, arginine, glutamine and proline in gestating and

lactating mammals, and aspartate and glutamate in enterally fed rats (Kim and Wu 2004; Li et al. 2007; Marc Rhoads and Wu 2009; Shewchuk et al. 1997; Wang et al. 2014a; Wu 2013b; Wu et al. 2008).

Glycine, also known as aminoacetic acid, has the simplest chemical structure among amino acids in nature, and has no D- or L- chemical configuration since it lacks an asymmetric carbon (Hall 1998; Jackson 1991; Wang et al. 2013a). The long history of glycine research dates back to 1820 when it was first obtained from acid hydrolysates of protein (i.e., gelatin) by the French chemist H. Braconnot. Subsequently, glycine was isolated from gelatin and meat by using alkaline hydrolysis. Glycine derived its name from its sweet taste, because the Greek word "*glykys*" means sweet (Meister 1992).

Glycine is one of the most abundant amino acids in animal proteins, and comprises one-third of amino acids in collagen and elastin (Wang et al. 2013a; Wu et al. 1999). Of note, glycine is the most abundant amino acid in plasma of postnatal pigs (Wu 2013c). Traditionally, glycine was classified as a NEAA in mammals since it can be synthesized from serine, threonine, and choline (Chao et al. 1953; Shemin 1946; Soloway and Stetten 1953; Tressel et al. 1986). However, the metabolic demand for glycine is considerable (Wang et al. 2013). The amount of endogenously synthesized glycine can meet, at most, only 50% of requirements for global protein synthesis (Gersovitz et al. 1980; Jackson 1991; Yu et al. 1985). For example, in poultry, the rate of endogenous glycine synthesis is much lower than the rate of utilization (Jackson 1991). Thus, studies have shown that endogenous synthesis of glycine is insufficient for supporting optimal metabolic requirements in rats, chickens and turkeys, and young pigs (Jackson 1991; Melendez-

Hevia and De Paz-Lugo 2008; Wang et al. 2014a; Wu et al. 2013). Lack of glycine can lead to long-term negative effects on the growth of animals and the health of their multiple organ systems, including the gastrointestinal (GI) tract disorders, oxidative stress, neurological damage, muscular dysfunction, and abnormal fetal development (Hernandes and Troncone 2009; Lewis et al. 2005; Li et al. 2016; Matilla et al. 2002; Wang et al. 2014a; Wu et al. 1994). Taken together, glycine should be classified as a conditionally essential amino acid for mammals and birds (Wu 2013c).

Glycine synthesis in animals

Glycine synthesis from serine

The serine-glycine pathway was the first pathway discovered for glycine biosynthesis. In 1946, D. Shemin found that serine could be rapidly converted into glycine, because ^{15}N and ^{13}C in serine were readily recovered in glycine (Shemin 1946). The conversion of serine into glycine is relatively simple since it is a one-step reaction. As shown in Figure 1.1, this reaction is catalyzed by tetrahydrofolate (THF)-dependent serine hydroxymethyl transferase (SHMT).

$$\text{Serine} + \text{H}_4\text{folate} \overset{\text{SHMT}}{\Longleftrightarrow} \text{Glycine} + 5,10\text{-methylene-H}_4\text{folate}$$

It is noteworthy that serine transfers one-carbon unit to tetrahydrofolate to form N^5-N^{10}-methylene-tetrahydrofolate (meTHF) (Wu 2013b). During the last 50 years, serine was considered the main substrate for glycine synthesis in animals. However, this view was formulated due to the limited knowledge of amino acid biochemistry and limited analytical tools at that time.

Serine hydroxymethyl transferase (SHMT) is a pyridoxal phosphate-dependent enzyme (Wang et al. 2013a). SHMT is located in both the mitochondria (mSHMT or SHMT2) and cytoplasm (cSHMT or SHMT1) of mammalian cells. For glycine synthesis, mSHMT is the primary enzyme in the cell, and the activity of mSHMT is much higher than that of cSHMT (Schirch and Peterson 1980). Narkewicz et al. (1996) reported that by eliminating mSHMT activity in Chinese hamster ovary (CHO) cells, glycine production decreased and serine accumulated (Narkewicz et al. 1996), which indicated that mSHMT is the major enzyme responsible for glycine synthesis. In contrast, cSHMT activity does not affect intracellular concentrations of glycine and serine. In those previous studies, alternative substrates for glycine synthesis were not examined.

There is evidence that total SHMT activity is regulated by nutritional status, hormones, and health conditions in a cell- and tissue-specific manner (Burns and Jackson 1982; Sanborn et al. 1975; Snell 1984; Snell et al. 1988). For example, folate availability limits SHMT activity (Burns and Jackson 1982). Sanborn et al. (1975) reported that estradiol and testosterone increased serine metabolism in the uterus and prostate gland via enhancing SHMT activity (Sanborn et al. 1975). Additionally, SHMT activity is much higher in tumor cells than in non-tumor cells, which may indicate a high glycine requirement for cells during tumorigenesis (Snell et al. 1988).

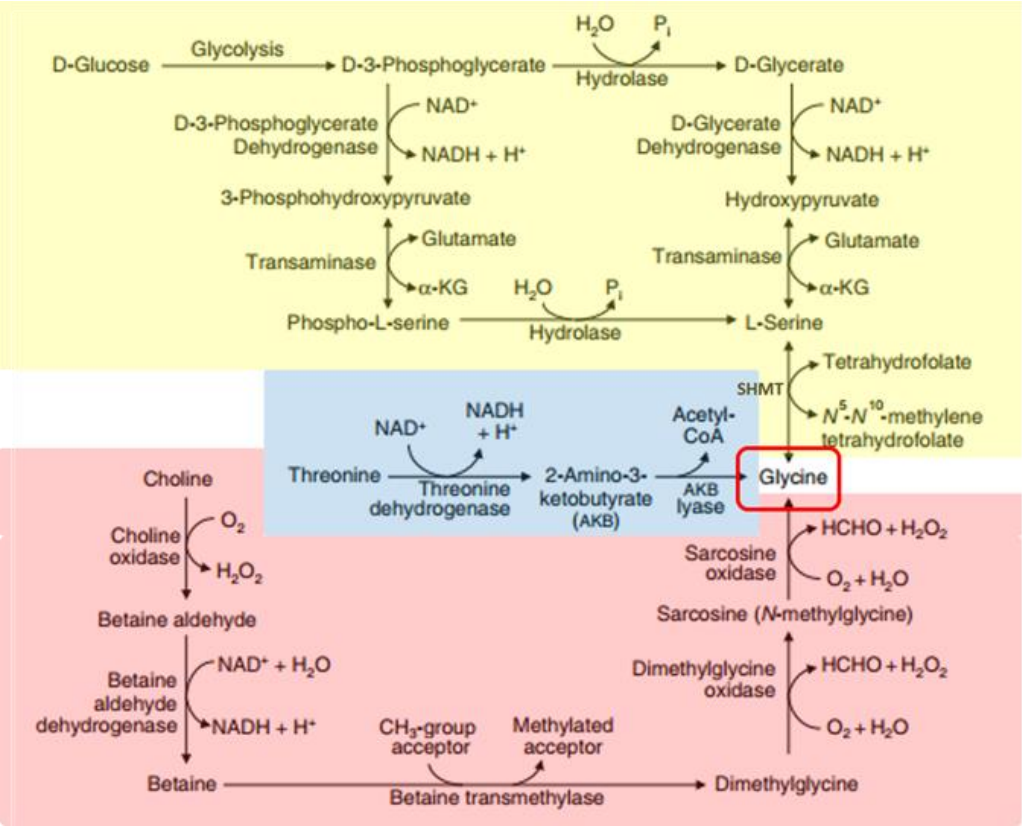


Figure 1.1. Classic pathway for the endogenous synthesis of glycine in animals. Glycine may be synthesized from serine, threonine, and choline. The three pathways play multiple physiological functions other than glycine synthesis. The yellow part of the figure highlights the serine-glycine pathway, the blue part the threonine-glycine pathway, and the red part the choline-glycine pathway. AKB, 2-Amino-3-ketobutyrate; α-KG, a-Ketoglutarate; SHMT, Serine hydroxymethyl transferase. Adapted and revised from Wang et al. (2013).

Glycine synthesis from threonine

For a long time, there was misidentification of the key enzyme that catalyzes the conversion of threonine into glycine. Schirch and Gross (1968) claimed that purified SHMT from rabbit liver not only catalyzed serine into glycine, but also cleaved threonine to glycine. Thus, threonine aldolase and SHMT were thought to be the same enzyme (Schirch and Gross 1968). However, subsequent work revealed that threonine aldolase is different from SHMT in bacteria and mammals. These two enzymes have different

functions and amino acid sequences, even though SHMT exhibits low threonine aldolase activity in rabbit liver (Bird and Nunn 1983; Dale 1978; Ogawa et al. 2000; Yeung 1986). There are two threonine-metabolic pathways: one requires threonine dehydrogenase and the other requires threonine aldolase. Both pathways can convert threonine into glycine, but the threonine dehydrogenase pathway accounts for most (more than 80%) degradation of threonine in animal livers (Bird and Nunn 1983; Davis and Austic 1994; Wu 2013a), but not all studies support this view. Darling et al. (2000) reported that the threonine dehydrogenase pathway played only a minor physiological role in adult humans (Darling et al. 2000). However, another study indicated that almost half of threonine degradation in newborn human infants occurs via the threonine dehydrogenase pathway (Parimi et al. 2005). This contradiction may indicate an age-dependent change in the expression of threonine-catabolic enzymes.

The major biochemical pathway for synthesis of glycine from threonine in the liver of animals requires two steps. In the first reaction, threonine and NAD^+ are converted by TDH into 2-amino-3-ketobutyrate (AKB) and NADH. In the second reaction, AKB is converted into glycine by AKB-lyase (Darling et al. 2000; House et al. 2001; Wu 2013b). Overall, the net reaction is as follows:

$$\text{Threonine} + \text{NAD}^+ + \text{CoA} \xrightarrow{\text{TDH \& AKB lyase}} \text{Glycine} + \text{Acetyl-CoA} + \text{NADH} + \text{H}^+$$

Glycine synthesis from Choline

Choline is a dietary ingredient in human and animal foods. The physiological functions of choline include one-carbon unit metabolism, cell membrane integrity, neurotransmission, and cell signaling, all of which are crucial for maintaining the normal function of cells (Zeisel 1981; Zeisel and Blusztajn 1994). The oxidative degradation of choline yields glycine via the formation of betaine (Soloway and Stetten 1953). Specifically, choline is oxidized to betaine by choline dehydrogenase and betaine aldehyde dehydrogenase in the mitochondria of liver and kidney cells (Zhang et al. 1992). Betaine transmethylase methylates betaine to form dimethylglycine. The latter is oxidized to sarcosine (N-methylglycine) by dimethylglycine oxidase. The sarcosine-glycine pathway occurs in a few tissues in animals and contributes to one-carbon unit metabolism (Bergeron et al. 1998; Yeo and Wagner 1994). The net reaction for glycine synthesis from choline is as follows:

$$\text{Choline} + \text{NAD}^+ + 4\text{O}_2 + 4\,\text{H}_2\text{O} \xrightarrow{\text{enzymes}} \text{Glycine} + 4\text{H}_2\text{O}_2 + 3\text{HCHO} + \text{NADH} + \text{H}^+$$

It should be born in mind that the contribution of choline to the synthesis of glycine is limited for two reasons. First, the amount of choline in both diet and physiological fluids is relatively low, compared to serine and threonine. Second, the choline-serine pathway is highly cell-specific and is utilized primarily for choline catabolism. Dietary choline is used mainly for the synthesis of phosphatidylcholine and sphingomyelin required for maintenance of cell membranes as) and for cholinergic neurotransmission (acetylcholine synthesis) (Wang et al. 2013a; Zeisel 1981).

Glycine facilitates inter-organ and intracellular transfer of methyl groups by bridging tetrahydrofolate (THF) and N^5-N^{10}-methylene-tetrahydrofolate (meTHF) in folate metabolism (Wu 2009; Wu 2013a; Wu 2013b). This is closely linked with the conversion of homocysteine (an oxidant) into methionine in the liver via *S*-adenosyl methionine (SAM)-dependent methylation (Finkelstein and Martin 2000).

Glycine catabolism in animals

Glycine catabolism through the glycine cleavage system

The glycine cleavage system (GCS) is the major enzyme system responsible for glycine catabolism in animals. GCS is a conservative enzyme complex widely distributed from prokaryotes to vertebrates (Kikuchi et al. 2008; Lowry et al. 1985b). In animals, the GCS is localized in the mitochondrial inner membrane and plays critical roles in glycine metabolism (Jois et al. 1992; Wang et al. 2013a).

$$\text{Glycine} + \text{NAD}^+ + \text{H}_4\text{folate} \overset{\text{GCS}}{\Longleftrightarrow} \text{NH}_3 + \text{CO}_2 + \text{NADH} + \text{H}^+ + 5,10\text{-methylene-H}_4\text{folate}$$

There are four components of the GCS: three enzymes designated as proteins P, T, and L, and one structural carrier protein, called protein H. Protein P is a pyridoxal phosphate-dependent protein or glycine dehydrogenase; protein T is amino-methyltransferase; protein L is known as dihydrolipoamide dehydrogenase; and protein H, the carrier protein, is a lipoic acid-containing protein (Kikuchi 1973; Kikuchi et al. 2008; Motokawa and Kikuchi 1969). Thus, a simple reaction for glycine deamination requires multiple enzymes for completion to ensure that errors leading to excess catabolism of glycine are minimized. The process of glycine degradation through the GCS complex consists of three reactions:

decarboxylation, amino-methyl transfer, and reoxidation of the reduced lipoate group (Figure 1.2). The glycine cleavage reaction is unlikely to be reversible under physiological conditions.

A number of studies have investigated the regulation of GCS activity in animals. Hormones, metabolic status, proteins, fatty acids, and concentrations of ions in plasma affect GCS activity (Jois et al. 1992; Lowry et al. 1985b; Mudd et al. 2007; Wu et al. 2014). Increasing the extracellular concentrations of glucagon or free Ca^{2+} enhances the activity of the glycine decarboxylase complex in mitochondria of the rodent liver (Jois et al. 1992). Metabolic acidosis also promotes the degradation of glycine via the GCS pathway in renal cortex tubule cells. The underlying mechanisms may include the accumulation of ammonia from glycine metabolism, which can aid in maintaining homeostasis of whole-body pH (Lowry et al. 1985b). Moreover, dietary protein intake activates glycine oxidation via GCS (Wang et al. 2013a).

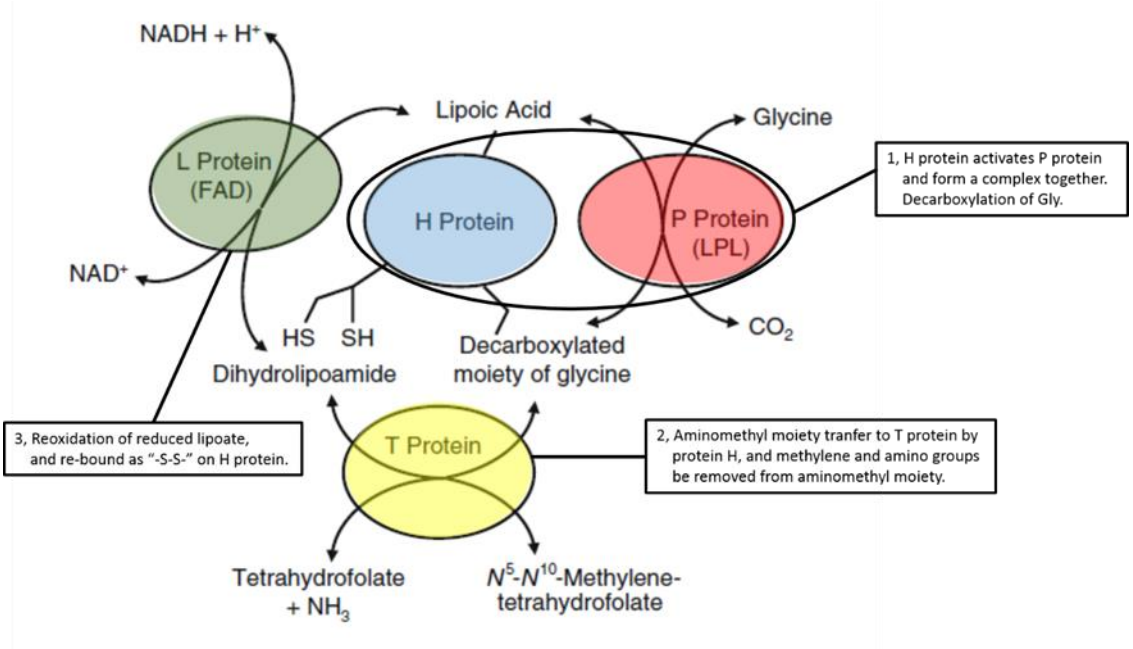


Figure 1.2. The glycine cleavage system (GCS). Protein P: glycine dehydrogenase, red; protein H: lipoic acid binding protein, blue; protein T: amino-methyl transferase, yellow; protein L: FAD-requiring dihydrolipoamide dehydrogenase, green.

Glycine catabolism through serine hydroxymethyl transferase

For glycine catabolism, SHMT is at the convergence of two key pathways: glycine metabolism and nucleotide/protein biosynthesis (Amelio et al. 2014). As noted previously, SHMT catalyzes the reversible reactions for serine and glycine catabolism in the body. Glycine catabolism provides serine and one-carbon units from N^5, N^{10}-methylene-tetrahydrofolate. The activity of SHMT is high in the ovary, placenta, fetal hepatocytes, and adult kidneys. This provides evidence for intracellular partitioning of serine and glycine metabolism from early to later in adult life, which is important for reproduction, fetal development, and survival (Lewis et al. 2005; Lowry et al. 1985b; Narkewicz et al. 1996; Thureen et al. 1995).

Glycine is utilized in different metabolic pathways via inter-organ coordination. Results from rats and pigs show a nonlinear relationship between glycine supplementation and the concentration of serine in plasma (Lowry et al. 1985b; Shoham et al. 2001; Wang et al. 2013a). It is likely that formation of serine from glycine is highly compartmentalized within cells and that glycine derived from serine is not released into the blood. This possibility, however, seems to be remote because only a small amount of serine is generated from glycine in cultured muscle cells (Sun et al. 2015) and enterocytes (Wang et al. 2014). Alternatively, folate availability limits the conversion of glycine into serine (Wang et al. 2013).

Quantitative analysis of endogenous glycine requirements for sow-reared pigs

The homeostasis of glycine is regulated by dietary intake, synthesis, and utilization of glycine. Some studies indicate that the amount of glycine from the diet and synthesis from serine, threonine, and choline is adequate for maximal weight gain (Sanborn et al. 1975; Schirch and Gross 1968).

Quantitative analysis of glycine intake and synthesis

The quantitative analysis of glycine intake and synthesis in sow-reared piglets is calculated based on results from previous studies (Wang et al. 2013). The amounts of glycine from milk and endogenous synthesis are summarized in Table 1.1.

Although sow's milk was traditionally thought to provide sufficient amino acids for supporting the optimal growth of neonatal piglets, sow-reared piglets grow at a suboptimal rate (Kim and Wu 2004; Wu 2010). Based on the quantitative analysis in Table 1.1, the

estimated intake of glycine from sow's milk provides, at most, only 20 % of the glycine required by the 7-day-old pig (Table 1.1). Therefore, at least 80% of the glycine needed by the neonate must be provided through endogenous synthesis.

For the last 50 years, serine has been considered as the main substrate for glycine synthesis in animals. However, serine conversion into glycine is limited. About 81% of serine in milk is utilized for protein synthesis and the diet provides at most 0.32 g of serine for glycine formation in young pigs (Wu 2010). The synthesis of glycine from choline and choline esters (glycerophosphocholine, phosphatidylcholine, and phosphocholine) provides about 36 mg/kg BW/day of glycine for sow-reared piglets (Donovan et al. 1997). Moreover, the amount of glycine synthesized from threonine is, at most, only 33 mg/kg BW/day in piglets. Thus, the amount of glycine from sow's milk and endogenous synthesis is about 456 mg/kg BW/day or nursing piglets.

Quantitative analysis of glycine requirements for growth and metabolic functions

Glycine has critical physiological functions in nutrition, metabolism, reproduction, and disease. About 80% of dietary glycine is used for protein accretion, and glycine accounts for 11.5% of total amino acids in the proteins of animal bodies (Wang et al. 2013a; Wu et al. 2013; Yan and Sun 1997). Therefore, young pigs have a particularly high requirement for glycine.

The average daily gain of a 7-day-old piglet is 200 g/day, and includes 27.2 g protein, which means that at least 1.22 g glycine is required to meet requirements for body weight gain of piglets (Wang et al. 2013a). As noted previously, glycine is the main substrate for

the synthesis of creatine (Brosnan et al. 2009), purine (Hall 1998; Hellwing et al. 2007), bile acid, heme (Wang et al. 2013a), and hepatic glutathione (Reeds et al. 1997). Based on those reports, the amount of glycine required for those metabolic functions is 189 mg/kg BW/day, as detailed in Table 1.1. Moreover, 96 mg glycine/kg BW/day is oxidized into ammonia and CO_2 in young pigs. Accordingly, the total amount of glycine required for protein accretion and metabolic functions is 1,204 mg/kg BW/day in sow-reared piglets.

Thus, there is an imbalance between glycine supply and utilization. The piglet must be able to synthesize 1.05 g glycine/kg BW/day from precursors other than serine, threonine, and choline. At present, the alternative pathways for glycine synthesis are largely unknown in neonatal piglets.

Hydroxyproline-glycine pathway in rodents and humans

Of particular interest, there is a strong link between glycine synthesis and hydroxyproline metabolism in rodents and humans. Hydroxyproline can be converted to glycine in the rodent liver (Ribaya and Gershoff 1979; Takayama et al. 2003) and kidneys (Lowry et al. 1985a), human liver (Baker et al. 2004) and human body (Knight et al. 2006; Melendez-Hevia et al. 2009). Published results indicate that the liver and kidney of mammals are the main sites for glycine synthesis from hydroxyproline. The specific biochemical process of the hydroxyproline-glycine pathway was proposed based on research with human colon cells, fibroblasts, and peripheral leukocytes (Cooper et al. 2008; Valle et al. 1979). The most direct evidence for the hydroxyproline-glycine pathway was reported by Ruiz-Torres and Kürten (1976) who found that after labeled 4-hydroxy-L-proline was administered to rats, 80% the labeled hydroxyproline was converted to glycine

within 1 hour. Those results suggested that free hydroxyproline was converted into glycine in animals (Ruiz-Torres and Kurten 1976). However, tissues other than liver and kidney that can synthesize glycine from hydroxyproline in any animal species is unknown. Based on previous studies, Wu (2013b) hypothesized that the hydroxyproline-glycine pathway exists in pigs (Figure 1.3) and that this pathway accounts for the majority of glycine synthesized in young pigs rather than synthesis of glycine from serine, threonine or choline (Wu 2013b).

Table 1.1. Quantitative analysis of the glycine balance (glycine from milk and endogenous synthesis versus the metabolic demands of nursing piglets) of 7-day-old sow-reared piglets. Adapted and revised from Wang et al. (2013).

	Amount of glycine (mg/kg BW per day)
Glycine from sow's milk	
Digestible milk intake (0.78 L/day; 1.2 g/L of whole milk)	311
Glycine from endogenous synthesis	
Glycine from dietary serine	81
Glycine from dietary threonine	31
Glycine from dietary choline	33
The maintenance, metabolic and growth requirements for glycine	
Body composition (protein & collagen)	1,216
Oxidation of "C" and "N" from glycine	96
Others metabolic molecules synthesis	
(including creatine, purines, bile acids, glutathione, and heme)	189
Unknown glycine synthesis sources	1045

The synthesis of glycine from hydroxyproline (Hyp) is an energy producing process. 1 mole of $FADH_2$ and NADH and 2 moles of pyruvate are produced during this process, which suggests that the conversion of hydroxyproline to glycine is preferred by animal bodies for glycine synthesis due to the production of both energy and glycine in this pathway. The first step in the pathway is oxidation of hydroxyproline, and that is the rate-limiting step in the synthesis of glycine from Hyp. The key enzyme, hydroxyproline oxidase (OH-POX) oxidizes Hyp into Δ^1-pyrroline-3-hydroxy-5-carboxylate (3-OH-P5C). 3-OH-P5C is degraded by Δ^1-pyrroline-5-carboxylate dehydrogenase and that same enzyme catalyzes the synthesis of Δ^1-pyrroline-5-carboxylate (P5C) (Valle et al. 1979). Gamma-hydroxyglutamate is produced from 3-OH-P5C, and serves as a substrate for synthesis of glyoxylate by glutamate-oxaloacetate transaminase (GOT) and 4-hydroxy-2-oxoglutarate aldolase (HOA) (Holmes and Assimos 1998). The final step in glycine synthesis is from glyoxylate by alanine: glyoxylate transaminase (AGT).

There are reasons for this biochemical process being the major pathway for glycine synthesis in the neonatal pig. First, there are abundant amounts of Hyp in sow's milk and tissues of piglets for synthesis of glycine. The glycine-proline-Hyp tripeptide (GPH) is the most abundant amino acid repeat (the basic structure unit) in collagen. There is a relatively high concentration of GPH in sow's milk. The concentration of Hyp (including peptide-bound and free forms) is about 8.9 mM on day 7 of lactation, and 1.5 times higher during early lactation than during later stages of lactation (Wang et al. 2013b). Second, the yield of ATP from this pathway requiring 1 mole of FADH2 and NADH and 2 moles of

pyruvate leads to the production of about 29 mole ATP. Thus, the Hyp-Gly pathway is energetically efficient for glycine production in the body.

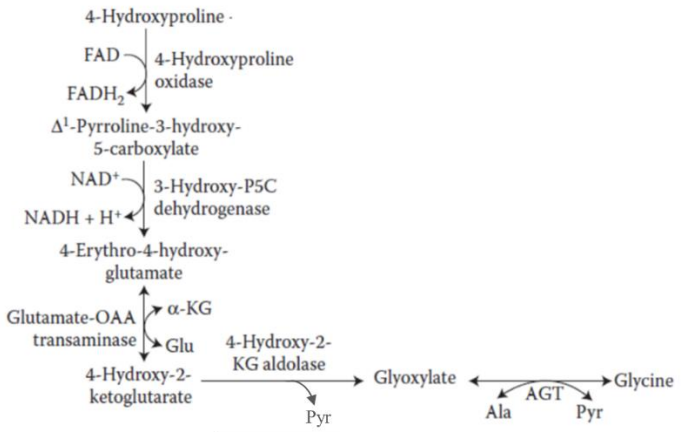


Figure 1.3. The proposed hydroxyproline-glycine pathway in neonatal pigs. Ala, Alanine; AGT, alanine:glyoxylate transaminase; α-KG, α-ketoglutarate; Glu, Glutamate; GOT, glutamate:oxaloacetate transaminase; P5C, Δ1-pyrroline-5-hydroxy-5-carboxylate; OAA, oxaloacetate; Pyr, pyruvate. Adapted and revised from Wu (2013b).

Physiological roles of glycine for growth

The physiological functions of glycine in animal growth and maintenance were ignored for a long time. Glycine was traditionally classified as a "non-essential amino acid" for mammals only because the endogenous pathways for synthesis of glycine *in vivo* was thought to just meet metabolic requirements by animals. However, growing evidence indicates that glycine has positive effects on improving animal growth and feed efficiency for lean tissue gain.

In animals fed a milk or plant-based diet, endogenous synthesis of glycine is insufficient to meet metabolic demands of the neonate (Wang et al. 2013a; Wu et al. 2014).

A large amount of glycine must be synthesized de novo to maintain homeostasis (Wang et al. 2013a). Because cell proliferation, protein synthesis, and nutrient utilization all depend on nucleic acid synthesis from glycine, glutamine, and folate, glycine is critical for animal growth, development, and health.

Glycine supplementation stimulates growth in animals

As a conditionally essential amino acid, glycine supplementation may be effective in improving nitrogen balance in animals. Results of recent studies indicate that endogenous synthesis of glycine does not meet metabolic requirements of sow-reared or post-weaning piglets, and that exogenous glycine increases daily weight gain and feed efficiency of growing pigs (Powell et al. 2011; Wang et al. 2014a; Wu et al. 2013). For example, Wang et al. (2014) reported that dietary supplementation with 2% glycine for 2 weeks increased average daily weight gain in milk-fed piglets, compared with the control piglets (Wang et al. 2014a). Powell et al. (2011) found that dietary supplementation with 0.52% glycine enhanced average daily weight gain and gain:feed intake ratio in 20-50 kg pigs fed a low-protein diet (13% crude protein). These results indicate that the amount of glycine provided from endogenous synthesis plus the low-protein diet cannot support maximum growth performance in pigs (Powell et al. 2011).

For avian species, glycine synthesis cannot meet metabolic demands since it is one of the amino acids used for synthesis of uric acid in the liver. Glycine supplementation to neonatal chickens may decrease muscle protein degradation and inhibit the expression of proteolytic-related genes, by suppressing concentrations of corticosterone in plasma

(Nakashima et al. 2008). Additionally, for growing chicks, at least 2.44% glycine plus serine is required in 16% crude protein diets (Dean et al. 2006). Corzo et al. (2004) suggested that dietary glycine should be at least 0.98% for 7- to 28-day-old broiler chicks to grow maximally (Corzo et al. 2004).

The roles of glycine in protein accretion in skeletal muscle

The accumulation and turnover of skeletal muscle are important for animal growth and meat production, since it is a large mass in the animal body which accounts for 40 ~ 45% of total body weight (Wu 2013b). Skeletal muscle has an anabolic response to glycine in various animal species, and the modulatory effects of glycine on muscle may result from the regulatory roles of glycine in activating the MTOR signaling pathway and one-carbon unit metabolism (Ham et al. 2016; Koopman et al. 2017; Ost et al. 2015; Sun et al. 2016a). Specifically, glycine supplementation promotes protein accretion (increasing protein synthesis and decreasing protein degradation) in the mouse myoblast cell line (C2C12). Expression of MTOR signaling pathway proteins, p-Akt, p-MTOR, p-$P70^{S6K}$, and p-4EBP1 were enhanced, and AMPK (an inhibitor of MTOR pathway) pathway was down-regulated in response to supplemental dietary glycine. The rate of protein synthesis was increased, and the rate of protein degradation was decreased when the concentration of glycine increased from 0 to 1 mM (Sun et al. 2016a). With the improvement in protein synthesis, muscle mass will increase. Glycine can restore muscle mass under conditions of inflammation by: 1) inhibiting proteolysis and attenuating protein loss (Ham et al. 2016); 2) maintaining Akt-MTOR-FOXO1 signaling; and 3) suppressing the activation of TLR4 and/or NOD2 signaling pathways (Liu et al. 2016).

The protective roles of glycine on the GI tract

The gastrointestinal tract is the main site digestion and absorption of components of the diet in animals, and optimal health of the GI tract is a prerequisite for optimal growth and production in animals. Accumulating evidence indicates that there is a positive effect of glycine on gastrointestinal health and development. First, glycine is a cytoprotective factor for prevention of enterocyte damage in both *in vitro* and *in vivo* models. The protective effects of glycine on ischemia-reperfusion (IR) injury in the small intestine are well-documented in humans, pigs, and rodents (Wang et al. 2013). The underlying mechanisms may relate to up-regulation of glycine transporter (GLYT1), down-regulation of cell death signaling, such as Bax and caspase-3, in the intestinal mucosa (Jacob et al. 2003). Second, glycine is highly effective in protecting the small intestine from inflammation and oxidative stress in humans and animals (Howard et al. 2010; Lee et al. 2002; Li et al. 2016; Razak et al. 2017; Tsune et al. 2003; Wang et al. 2014b). The anti-oxidative roles of glycine in the GI tract are achieved, in part, through glutathione synthesis. GSH is capable of scavenging reactive oxygen species (ROS), free radicals, peroxides, and lipid peroxides by either directly removing free radicals, or by reducing ROS. This physiological process is very efficient in enterocytes (Howard et al. 2010; Wang et al. 2014b; Wu 2013b). Additionally, glycine supplementation to lipopolysaccharide (LPS)-pretreated rats can increase their survival rate by decreasing tumor necrosis factor alpha (TNFA) and other pro-inflammatory cytokines in the small intestine (Ikejima et al. 1996). There is also evidence for the protective roles of glycine in tumor formation and growth in the intestine (Amin et al. 2003; Tsune et al. 2003). Third,

physiological concentrations of glycine help to maintain the intestinal mucosal barrier by modulating the expression and distribution of mucosal barrier proteins [(claudin-7 and tight junction 3 protein (ZO-3)] in intestinal epithelial cells (Li et al. 2016), because the intestinal mucosal barrier is critical for GI health, nutrient absorption, and utilization (Wang et al. 2015).

Heme synthesis and one carbon metabolism

The pathway of heme synthesis from glycine was discovered in the 1940s (Radin et al. 1950; Shemin et al. 1948). The heme-containing hemoprotein carries oxygen in red blood cells in blood of animals, and the heme-containing myoglobin stores oxygen in muscle for mitochondrial respiration and biogenesis (Wang et al. 2013a; Wu 2009). The family of hemoproteins includes hemoglobin, myoglobin, cytochromes, guanylate cyclase, NADH dehydrogenase, and coenzyme Q reductase (Poulos 2014). Cytochromes, NADH dehydrogenase, and coenzyme Q reductase play essential roles in energy supply and mitochondrial biogenesis (Dai et al. 2013; Wu et al. 2014). Another function of heme is to produce carbon monoxide (CO). CO, like nitric oxide (NO), is an endogenous cellular gas signaling molecule, and has important regulatory functions *in vivo* (Dai et al. 2013; Wang et al. 2013a). By triggering cGMP formation, CO induces protein phosphorylation reactions to regulate energy balance and smooth muscle relaxation (Wu 2013a). Moreover, CO can modulate immune system functions and neurotransmission (Wu and Wang 2005).

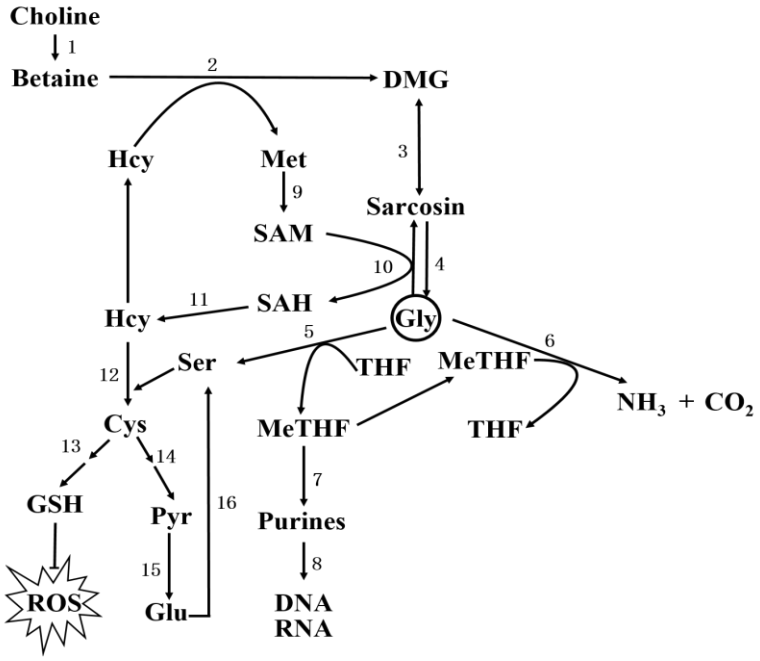


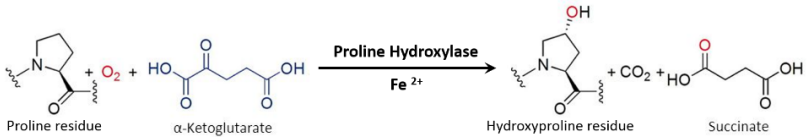
Figure 1.4. The roles of glycine in one carbon unit metabolism in cells. Betaine, choline, cysteine, glucose, homocysteine, serine, and tetrahydrofolate participate in the transfer of one-carbon units for multiple physiological functions in animals. Enzymes and co-factors involved are: (1) choline dehydrogenase; (2) betaine:homocysteine methyltransferase and vitamin B_{12}; (3) dimethylglycine oxidase; (4) sarcosine oxidase; (5) serine hydroxymethyl transferase; (6) glycine cleavage system; (7) formyltransferase; (8) enzymes for DNA and RNA synthesis; (9) S-adenosylmethionine synthase; (10) S-adenosylmethionine, a major methyl group donor in methyltransferase reactions,; (11) S-adenosyl-homocysteine hydrolase; (12) cystathionine β-synthase and cystathionine γ-lyase; (13) γ-glutamyl-cysteine synthetase and glutathione synthetase; (14) a sequential series of enzymes that convert cysteine to pyruvate; (15) enzymes required for gluconeogenesis; and (16) a sequential series of enzymes that convert glucose to serine. Cys, cysteine; DMG, dimethylglycine; GSH, glutathione; Glu, glucose; Gly, glycine, Hcy, homocysteine; Met, methionine; meTHF, N^5-N^{10}-methylenetetrahydrofolate; ROS, reactive oxygen species; SAH, S-adenosylhomocysteine; SAM, S-adenosyl-methionine; Ser, serine; THF, tetrahydrofolate; Pyr, pyruvate.

The understanding of physiological functions and regulation of cellular one-carbon unit metabolism is limited. The core role of one-carbon unit metabolism is transferring one-carbon units from specific amino acids to different target sites for multiple physiological functions. There are two main pathways for one-carbon unit metabolism (Figure 1.4): the folate-related pathway and the methionine-related pathway. The glycine synthesis and catabolism pathways are involved in one-carbon unit transfer, which is

important in nutrient metabolism, epigenetic regulation, mitochondrial biogenesis, cellular redox state balance, and oncogenesis (Ducker and Rabinowitz 2017; Locasale 2013). Recent genetic, functional, and clinical evidence indicates a link between cancer pathogenesis and glycine metabolism (Locasale 2013).

Metabolism and functions of hydroxyproline/proline

Different from other α-amino acids in chemical structure, proline (Pro) and hydroxyproline (Hyp) are unique as imino acids (Wu 2013b). Hydroxyproline, primarily 4-hydroxy-L-proline, is found in acid hydrolysates of gelatin. Hydroxyproline is not a proteinogenic imino acid, but a product of the post-translational hydroxylation of proline (Hausmann and Neuman 1961). The structures of Pro and Hyp, and the conversion from Pro to Hyp are as follows.



Proline residues in proteins (mainly collagen) can be hydroxylated to either 4- or 3-hydroxy-L-proline residues in the endoplasmic reticulum of cells of most animals (Gorres and Raines 2010; Krane 2008). However, the production and amounts of 3-hydroxy-L-proline are limited *in vivo*. The abundance of 4-hydroxy-L-proline residues is approximately 100 times greater than 3-hydroxy-L-proline residues in animal collagen (Krane 2008; Wu et al. 2011).

Physiological functions of Pro and Hyp

Proline and hydroxyproline play versatile physiological roles in animal maintenance, growth, reproduction, tumorigenesis, and anti-oxidation, especially for young animals (Wu et al. 2017). First, Pro and Hyp are abundant in collagen. Pro and Hyp constitute approximately 30% of the amino acids in whole-body collagen (Wu et al. 2011). There are reports that dietary supplementation with Hyp or proline improves collagen synthesis and growth of animals, including pigs, chickens, and fish (Li and Wu 2017). However, the anti-oxidative and apoptosis-modulatory roles of Hyp have been demonstrated in recent years (Wu et al. 2017). Hyp can scavenge free radicals and produce reactive oxygen species (ROS) for regulation of cellular redox status (Cooper et al. 2008; Phang and Liu 2012; Wu et al. 2011). The key enzyme for Hyp catabolism, hydroxyproline oxidase (OH-POX), mediates the caspase-9 pathway, and induces apoptosis of cells (Cooper et al. 2008). There is a suggestion that proline degradation could be an alternative energy source for ATP generation in cells with a limited supply of nutrients (Pandhare et al. 2009). Second, there is more and more evidence indicating that proline has vital regulatory roles in protein turnover by activating the MTOR signaling pathway in young animals. Wu et al. (2011) reported that dietary supplementation with 2% proline enhanced body weight gain in weanling piglets. Compared with the low-protein diet, supplementing 9% proline to an enteral diet had positive effects on protein accretion in the liver, muscle, small intestine, and skin of neonatal miniature piglets (Brunton et al. 2012). Third, by producing polyamines via proline oxidase, ornithine aminotransferase, and ornithine decarboxylase in placentae and the neonatal small intestine, proline supports their rapid growth through

stimulating protein synthesis and cell proliferation. These effects of proline provide a biochemical basis for using proline to reduce intrauterine growth restriction (IUGR) in mammals (Wu et al. 2008).

Summary and objectives

Glycine accounts for 11% of total amino acids in animal proteins, and can be synthesized in the liver from dietary serine, threonine, and choline. Thus, glycine was traditionally classified as a non-essential amino acid. However, there is increasing evidence that glycine has versatile roles to support growth and health, metabolic regulation and cytoprotection, as well as serve as an antioxidant and anti-inflammatory amino acid in animals. The nutritional significance of glycine in one-carbon unit metabolism can be extended to epigenetic regulation (DNA or protein methylation), mitochondrial biogenesis, and cellular redox signaling. The amount of endogenous glycine synthesized is not sufficient to meet metabolic needs of animals (including young pigs); therefore, dietary supplementation of glycine is required to optimize health and growth of piglets. The concentrations of glycine in plasma of IUGR piglets suggest that glycine is deficient in low-birth-weight piglets (<1.1 kilograms, IUGR). Based on these results, glycine should be categorized as a conditionally essential amino acid for animals.

Glycine from milk meets only 23% of requirements for protein synthesis in neonatal piglets. Moreover, the contributions of serine, threonine, and choline to glycine synthesis from provide only 11% of the piglet's total glycine requirement (Wang et al. 2013a; Wu and Knabe 1994; Wu et al. 1999). At least 0.71 g glycine/kg BW/day is needed for tissue

protein synthesis. There are reports that hydroxyproline is converted into glycine in the liver and kidney of rodents and in the human body. However, it is unknown whether other tissues possess the capacity for synthesizing glycine from hydroxyproline. Furthermore, nothing is known about this metabolic pathway in any tissues of farm animals, including pigs.

The central hypothesis of this dissertation is that hydroxyproline is a major precursor for glycine synthesis in sow-reared pigs. Moreover, glycine deficiency contributes to the suboptimal growth and high mortality in IUGR piglets, and the glycine deficiency can be ameliorated by dietary supplementation with glycine. The overall objectives of this dissertation research were to: 1) identify novel endogenous pathways for the synthesis of glycine in neonatal pigs; 2) explain the basis for low concentrations of glycine in the plasma of IUGR pigs; and 3) define the nutritional role of glycine in neonatal pigs.

CHAPTER II

GLYCINE SYNTHESIS FROM HYDROXYPROLINE IN TISSUES OF NEONATAL PIGS

The present study was conducted to investigate glycine synthesis from 4-hydroxyproline in tissues of sow-reared pigs. At 0, 7, 14 and 21 days of age, 6 piglets were sacrificed for each age group and their tissue samples were obtained for metabolic studies, as well as the determination of amino acids, activities of glycine-synthetic enzymes, protein localization, and gene expression. Our results indicated that at birth, hydroxyproline oxidase (OH-POX), proline oxidase (POX), alanine:glyoxylate transaminase (AGT), and 4-hydroxy-2-oxoglutarate aldolase (HOA) (key enzymes for glycine synthesis from hydroxyproline) were present in the liver and kidneys. Except for AGT, all of those enzymes were absent from skeletal muscle, heart, small intestine, and pancreas. At all ages, AGT was present in all the tissues, but the heart. The activities of the glycine-synthetic enzymes were decreased in the liver and kidneys between days 0 and day 21, but increased in the pancreas and small intestine over time ($P < 0.05$). Similar results were obtained for expression of mRNAs for those enzymes. For serine-glycine and threonine–glycine pathways: the enzymatic activities and expression of mRNAs for serine hydroxymethyl transferase (SHMT) and threonine dehydrogenase (TDH) increased from day 0 to day 21 of age in most tissues ($P < 0.05$). The localization of OH-POX and POX shifted from the periportal to perivenous hepatocytes in the liver as the piglets grew. With increasing age, the abundances of OH-POX and POX decreased in the liver, but increased

in the pancreas, small intestine, and stomach. Skeletal muscle and gallbladder expressed OH-POX and POX, but the abundances of those two proteins did not change with age. Tissues incubated with 0 to 5 mM hydroxyproline synthesized glycine in a concentration-dependent manner, but the conversion of glycine into serine was limited. These novel findings indicate that hydroxyproline, an abundant metabolite in sow's milk and a product of collagen degradation, is an important substrate for glycine synthesis in young pigs, instead of serine and threonine.

Introduction

Glycine is one of the most abundant amino acids in animals and comprises about 30% of the collagen and elastin in the body (Wu 2009; Wu et al. 2014; Wu et al. 1999). Glycine participates in the formation of N^5-N^{10}-methylenetetrahydrofolate from N^5-methyltetrahydrofolate and, thus, plays an important role in one-carbon unit metabolism (Jackson 1991; Mudd et al. 2007; Wang et al. 2013a). With the action of glycine N-methyltransferase, glycine reacts with S-adenosyl methionine (SAM) to form sarcosine and S-adenosyl-homocysteine, thereby affecting the availability of SAM for DNA and protein methylation (Mudd et al. 2007). Glycine can be synthesized from serine (Shemin 1946), threonine (Chao et al. 1953), and choline (Soloway and Stetten 1953) via well-established pathways. Serine hydroxymethyltransferase (SHMT) catalyzes the reaction of serine to glycine, whereas threonine dehydrogenase (TDH) initiates the degradation of threonine to yield glycine. Furthermore, glycine is synthesized from choline via a series of reactions involving sarcosine formation.

Results of recent studies have shown that glycine from milk meets only 23% of the requirements of neonatal piglets for protein synthesis (Wu 2013c). Moreover, serine, threonine, and choline provide, at most, only 11% of the piglet's total glycine requirement (Wang et al. 2013a; Wu and Knabe 1994; Wu et al. 1999). Clearly, additional substrates exist for glycine synthesis in piglets.

There are reports that hydroxyproline can be converted to glycine in the liver (Ribaya and Gershoff 1979; Takayama et al. 2003) and kidney (Lowry et al. 1985a) of rodents, as well as liver (Baker et al. 2004) and body tissues of humans (Knight et al. 2006). The specific biochemical processes of the hydroxyproline-glycine pathway were also elucidated in these species (Cooper et al. 2008; Knight et al. 2006; Lowry et al. 1985a; Melendez-Hevia et al. 2009; Takayama et al. 2003; Valle et al. 1979). However, little is known about hydroxyproline metabolism in newborn pigs. Therefore, the present study was conducted to determine if hydroxyproline is the major substrate for glycine synthesis in tissues of sow-reared pigs. To achieve this goal, a series of experiments were performed to determine enzymatic activities, expression of mRNAs and proteins for key enzymes, cell-specific localization of proteins, and direct quantification of glycine production from 4-hydroxyproline.

Materials and methods

Pigs

The experimental protocol of this study was approved by the Texas A&M University Institutional Animal Care and Use Committee.

Piglets were the offspring of Yorkshire x Landrace sows and Duroc boars. They were maintained at the Texas A&M University Swine Center. The average litter size of sows was 12.4 at weaning. Throughout the gestation and lactation periods, sows had free access to water and corn-soybean meal based diets that met NRC requirements (NRC 2012). On days 0, 7, 14, and 21 of age, six piglets for each day of age with average body weights of 1.41 ± 0.12, 3.13 ± 0.31, 4.03 ± 0.18, and 5.51 ± 0.34 kg were randomly selected and euthanized, as described previously (Hu et al. 2015).

Collection of tissues collection from piglets

At 0, 7, 14 and 21 days of age, after blood sampling, piglets were anesthetized with an intramuscular injection of Telazol (10 mg/kg body weight) and then euthanized by intracardiac administration of saturated KCl. After the abdomen was opened, the heart, liver, lung, stomach (with luminal contents being removed), pancreas, spleen, jejunum (proximal half of the remaining small intestine, with luminal contents being removed), kidneys, gallbladder, skeletal muscle, skin and brain were quickly isolated and weighted (Wang et al. 2014c). Tissues were cut into small portions, and some were fixed in freshly prepared 4% (wt/vol) paraformaldehyde in PBS (pH 7.2) for 24 h, followed by storage in 70% ethanol for 24 h. The fixed tissues were dehydrated through a graded series of alcohol to xylene and embedded.

Slices of fresh tissues were placed in ice-cold Krebs-Henseleit buffer (KHB) before being used in explant cultures (see below). The remaining portions of the tissues were frozen in liquid nitrogen and stored at -80°C.

Analysis of enzymatic activities of tissues

Tissue homogenate. Frozen tissue (0.5 g) was homogenized in 2 mL of freshly prepared buffer consisting of 300 mmol/L sucrose, 5 mmol/L HEPES (pH 7.4), 1 mmol/L EDTA, 3 mmol/L dithiothreitol, 0.5% (v:v) Triton X-100, and 0.1% (wt:v) protease inhibitor (aprotinin, chymostatin, pepstatin A, and phenylmethylsulfonyl fluoride; 5 µg/mL each) for 2 min on ice (Lowry et al. 1985). The whole homogenization mixture was transferred to a tube, which was centrifuged at 600 x g for 10 min at 4°C. The supernatant fluid was subjected to three cycles of freezing in liquid nitrogen and thawing in a 37°C water bath before use for enzyme assays (Carnie et al. 1982; Lowry et al. 1985a; Lowry et al. 1985b).

Activity of hydroxyproline oxidase. The assay for hydroxyproline oxidase was performed as described by Carnie et al. (1982) with modifications Briefly, the assay solution (3 mL) consisted of 40 mM KPi (pH 8.0), 150 mmol/L hydroxyproline, 8 µmol/L cytochrome C (C2506; Sigma-Aldrich), and 0.3 mL of tissue homogenate. The solution was incubated at 37°C. After 0 and 30 min, 0.8 mL of the mixture was added to 1.6 mL of 40% ethanol and 6% trichloroacetic acid (TCA) which contained 1 mg/mL *O*-aminobenzaldehyde (A9628; Sigma-Aldrich). The mixture was allowed to stand for 30 min at room temperature, and centrifuged at 600 x g for 10 min to acquire the supernatant fluid for measurement of optical density (OD) at the wavelength of 443 nm. Buffer (80 mmol/L KPi buffer, pH 8.0) was used instead of 600 mmol/L hydroxyproline in the blank tube (Carnie et al. 1982; Lowry et al. 1985a). The enzymatic activity was calculated according to the following formula:

$$\frac{(\Delta A_{443} / \text{min for sample} - \Delta A_{443} / \text{min for blank}) \times 3.75 \times 1000}{2.59 \times \text{Tissue weight (g)} \times \text{Reaction time (min)}}$$

Activity of proline oxidase. The activity of proline oxidase in different tissues was measured as described by Wu et al. (1997). Briefly, the reaction mixture contained 30 mmol/L proline, 0.2 mmol/L cytochrome C, 50 mmol/L KPi buffer (pH 7.5), and tissue homogenate. The solution was incubated at 37°C for 30 min, and then 10% TCA was added to stop the reaction, followed by the addition of 0.1 mL of 100 mmol/L *O*-aminobenzaldehyde. The mixture was allowed to stand for 30 min at room temperature and then centrifuged at 600 x g for 10 min. The supernatant fluid was used for measurement of OD at the wavelength of 440 nm. In the blank, 10% TCA was added to the assay mixture before incubation (Herzfeld et al. 1977). The enzymatic activity was calculated according to the following formula:

$$\frac{(\Delta A_{440} / \text{min for sample} - \Delta A_{440} / \text{min for blank}) \times 1.60 \times 1000}{2.70 \times \text{Tissue weight (g)} \times \text{Reaction time (min)}}$$

Activity of 4-hydroxy-2-oxoglutarate aldolase. The activity of 4-hydroxy-2-oxoglutarate aldolase in tissues was determined as previously described (Carnie et al. 1982). Briefly, the reaction solution (2 ml) contained 0.2 mL of homogenate 100 mmol/L DL-4-hydroxy-2-ketoglutarate (disodium salt), and 100 mmol/L KPi buffer (pH 7.8). The mixture was incubated at 37°C for 20 min, and 10% TCA was added to stop the reaction. After the solution was centrifuged at 600 x g for 10 min, the supernatant fluid was used to measure pyruvate as previously described (Li et al. 2009). Buffer (100 mmol/L KPi buffer,

pH 7.8) was used instead of 100 mmol/L DL-4-hydroxy-2-ketoglutarate (disodium salt) in the blank group.

Activity of alanine: glyoxylate transaminase. The activity of alanine:glyoxylate transaminase was determined by quantifying pyruvate production, as described by Rowell et al. (1972). Briefly, the assay solution (0.5 mL) contained 0.02 mL of tissue homogenate, 100 mmol/L glyoxylic acid, 100 mmol/L alanine, and 100 mmol/L KPi buffer (pH 7.4). The mixture was incubated at 37°C for 20 min, and 10% TCA was added to stop the reaction. The solution was centrifuged at 600 x g for 10 min. The supernatant fluid was analyzed for pyruvate, as previously described (Rowsell et al. 1972). Buffer (100 mmol/L KPi buffer, pH 7.4) was used instead of 100 mmol/L glyoxylic acid in the blank tube.

Activity of threonine dehydrogenase. Threonine dehydrogenase was analyzed by measuring NADH accumulation as described by Chen et al. (2009). Briefly, 0.02 mL of homogenate was added to a mixture of 100 mmol/L Tris/HCl (pH 8.4), 5 mmol/L NAD, 0.125 mmol/L pyridoxal phosphate, and 50 mmol/L threonine. The solution was incubated at room temperature for 10 min, and OD values read at the wavelength of 340 nm using a microplate reader. Buffer (100 mmol/L Tris/HCl, pH 8.4) was used instead of 50 mmol/L threonine in the blank tube.

Serine hydroxymethyltransferase. The assay solution consisted of 0.1 mL of tissue homogenate, 20 mmol/L L-serine, 0.36 mmol/L tetrahydrofolic acid, and 100 mmol/L KPi buffer (pH 7.5). The mixture was incubated at 37°C for 20 min. The reaction was terminated by addition of 0.2 mL of 1.5 mol/L $HClO_4$, followed by addition of 0.1 mL of

2 mol/L K_2CO_3. After being centrifuged at 10,000 rpm for 2 min, the supernatant fluid was used for analysis of glycine using high performance liquid chromatography (HPLC) (Hu et al. 2015; Wu et al. 1997; Wu and Knabe 1994; Wu and Meininger 2008).

Pyruvate determination. Pyruvate, a product of HOA and AGT, was determined using lactate dehydrogenase, as described by Wu et al. (1995). Briefly, 0.2 mL pyruvate standard or sample was mixed with 0.15 mL of 50 μM NADH and 1.5 mL 150 mM phosphate buffer (pH 7.5). After a 2 min incubation at room temperature, fluorescence was measured at the excitation wavelength of 340 nm and an emission wavelength of 460 nm in a Molecular Devices Spectra Max M2 microplate reader. Then, 5 μL of L-lactate dehydrogenase was added to the assay well. After a 5 min incubation at room temperature, fluorescence was measured again. The difference in fluorescence before and after the addition of lactate dehydrogenase was used to calculate concentrations of pyruvate on the basis of the pyruvate standard curve.

RNA isolation and quantitative Real-Time PCR analyses

Total RNA was isolated from piglet tissues using Trizol (15596026; Invitrogen) according to Jobgen et al. (2009). The quantity and quality of the total RNA were determined by spectrometry (wavelength 230 nm). The expression of mRNAs for hydroxyproline oxidase (PRODH2) (Summitt et al. 2015), 4-hydroxy-2-oxoglutarate aldolase (HOGA), alanine-glyoxylate aminotransferase (AGXT2), proline oxidase (PRODH1), serine hydroxymethyltransferase (SHMT2), and L-threonine dehydrogenase (TDH) in different tissues were determined by quantitative real-time polymerase chain

reaction. Primers were designed using the Primer Express Software Version 1.5 (Applied Biosystems). Primer pairs for selected genes are summarized in Table 2.1. The quantitative PCR was performed using the ABI prism 7900HT system (Applied Biosystems) with the Power SYBR Green PCR Master Mix (4309155; Applied Biosystems) as instructed by the manufacturer. Swine 18S ribosomal (RN18S, GenBank No. NR_046261) was used as the reference gene (Table 2.1). The abundance of mRNAs was calculated using the comparative Ct method (Wang et al. 2014d).

Immunohistochemical analyses

Immunohistochemical localization of OH-POX and POX proteins in piglet tissues was performed as described previously (Wang et al. 2014d) in tissue sections (~5 μm) with rabbit anti-OH-POX immunoglobulin G (IgG) (Adapted from Dr. James M. Phang, NIH), and rabbit anti-POX polyclonal IgG (HPA020361; Sigma-Aldrich) at a dilution of 1:500 and 1:200, respectively. Antigen retrieval was performed by boiling the sample in 0.01 M sodium citrate buffer, pH 6.0. Purified nonrelevant rabbit or mouse IgG was used as a negative control to replace the primary antibody at the same final concentration. Immunoreactive protein was visualized in sections using the VECTASTAIN ABC Kit (PK-6101 for rabbit IgG; Vector Laboratories) and 3,3′-diaminobenzidine tetrahydrochloride (D5637; Sigma-Aldrich) as the color substrate.

Table 2.1. Primer pairs sequence for selected genes

Gene	Accession number	Forward primer sequence (5' - 3')	Reverse primer sequence (5' - 3')
18S	NR_046261	CTTGCAGGGCGGTGACAG	GCCCTCGGTCGAGTTGTC
AGT	XM_013997641	GATCTACACGCGGAAGACC	GCTGTACAAGCCAATGACG
GOT 2	NM_213928	TATGTCACCGTGCAGACCAT	AGGAAGACATCTCGGCTGA
HOGA	NM_001190169	TGCACTCGTTTGGGAAACCT	AAGGGCTAGAATGCAGCTC
POX	NM_011172	GAGGACCAGGAGTCCATC	CAGGACTCCATCTCCTTG
PRODH 2	NM_021232	GTGACCATGTCTCCTTGGCA	CCGCAGTACACTTCGGTTC
SHMT 2	NM_021232	GAGAGTCTATCAGACAGTG	CGAGTACTTGTTGTTCAGA
TDH	NM_214004	CCAGAGCCGTGAACATCAC	AGAAGTGGGTCCAAAAGCC

Production of glycine by tissues incubated with hydroxyproline

The production of glycine in tissues incubated with 4-hydroxyproline was determined using an established method (Self et al. 2004; Wu et al. 1989). Slices of (~100 mg) freshly isolated tissues from piglets were incubated at 37^{o}C for 2 h in 2 mL oxygenated KHB buffer containing 5 mM D-glucose, 20 mM HEPES (pH 7.4), and 0, 0.1, 0.25, 0.5, 2, or 5 mM 4-hydroxyproline. The reaction was terminated by addition of 200 μL of 1.5 mol/L $HClO_4$. The tissue plus the incubation medium was homogenized for 2 min on ice, and the solution was neutralized with 100 μL of 2 mol/L K_2CO_3. After being centrifuged at 600 x g for 5 min at 4 °C, the supernatant fluid was used for HPLC analysis of amino acids (Wu and Meininger 2008). In parallel experiments, concentrations of amino acids before incubation were also determined as previously described.

Statistical analysis

Results are expressed as means ± SEM. Statistical analyses of data were performed by one-way analysis of variance using the General Linear Models procedures (Assaad et al. 2014). Differences among treatment means were determined using the Student-Newman-Keuls (SNK) multiple comparison method (Assaad et al. 2014). A probability value of $\leq$ 0.05 was taken to indicate statistical significance.

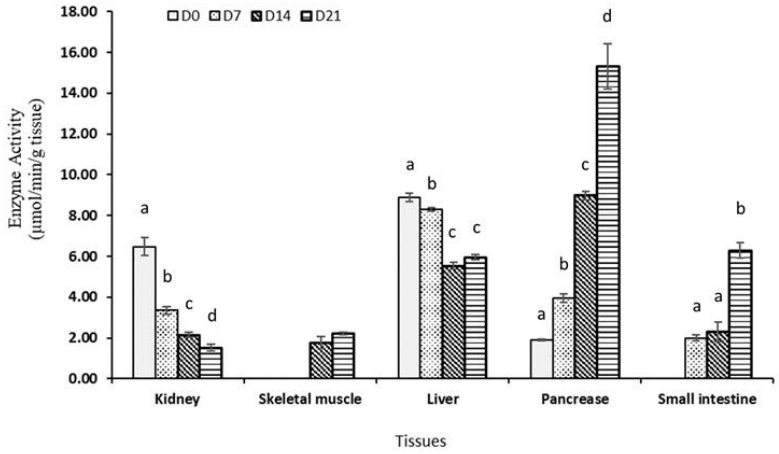
Results

Enzymatics activities of the hydroxyproline-glycine pathway

Figures 2.1 to 2.2 illustrate the enzymatic activities of OH-POX, POX, HOA, and AGT in tissues from piglets. OH-POX catabolizes the conversion of hydroxyproline into Δ^1-pyrroline-3-hydroxy-5-carboxylate (3-OH-P5C), which is a rate-controlling enzyme for the hydroxyproline-glycine pathway (Lowry et al. 1985a; Phang et al. 1981; Ruiz-Torres and Kurten 1976; Takayama et al. 2003; Valle et al. 1979; Wu et al. 2011). The activities of OH-POX in the liver and kidney decreased gradually during the first 3 weeks of postnatal life. OH-POX activity was not detected in the heart (day 0 to day 14), skeletal muscle (days 0 and 7), small intestine (day 0), or stomach (days 0-21). OH-POX activity in the pancreas and small intestine tended to increase between days 7 and 21 of age.

For comparison, the activity of POX, which oxidizes proline to Δ^1-pyrroline-5-carboxylate (P5C), was not detected in the heart (days 0 to 14), skeletal muscle (day 0), pancreas (day 0), or stomach (days 0 to 14). However, the liver and kidney had high POX

activity at birth, which decreased progressively as the piglet became older ($P < 0.05$). POX activity in the small intestine increased progressively with age.

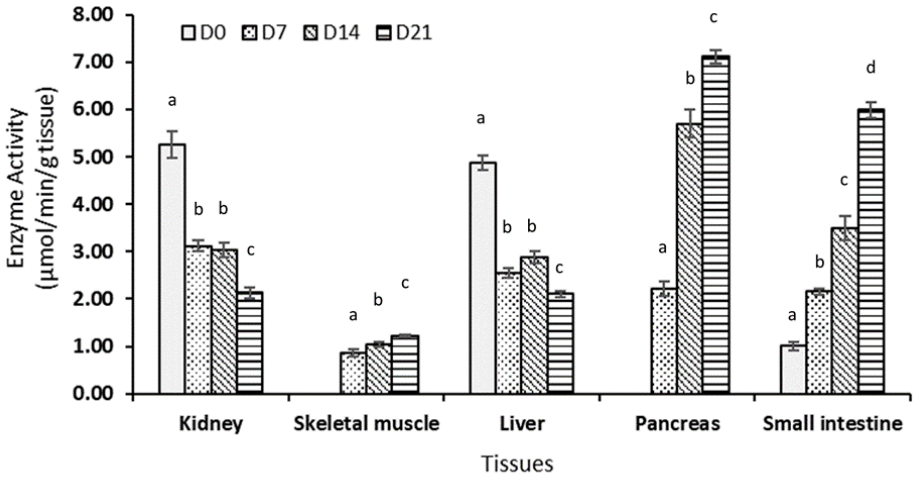


	Hydroxyproline oxidase (OH-POX) (µmole/min/g tissue)			
	Day 0	Day 7	Day 14	Day 21
Heart	-----	-----	-----	1.41 ± 0.13
Kidney	6.48 ± 0.43[a]	3.35 ± 0.18[b]	2.15 ± 0.12[c]	1.52 ± 0.18[d]
Skeletal Muscle	-----	-----	1.77 ± 0.20	2.21 ± 0.08
Liver	8.89 ± 0.21[a]	8.30 ± 0.07[b]	5.53 ± 0.17[c]	5.95 ± 0.14[c]
Pancreas	1.89 ± 0.04[a]	3.93 ± 0.21[b]	8.98 ± 0.22[c]	15.3 ± 1.20[d]
Small Intestine	-----	1.99 ± 0.14[a]	2.28 ± 0.47[a]	6.29 ± 0.39[b]

Figure 2.1. The enzymatic activity for hydroxyproline oxidase (OH-POX) in tissues from piglets at different ages (days 0, 7, 14 and 21). The results indicated that liver and kidney have the greatest hydroxyproline oxidase enzymatic activity as newborn piglets. Values are expressed as means ± SEM, n = 6 per time point. a-b means in a row without a common superscript letter differ ($P < 0.05$) as analyzed by one-way ANOVA and the Student–Newman–Keuls (SNK) multiple comparison test.

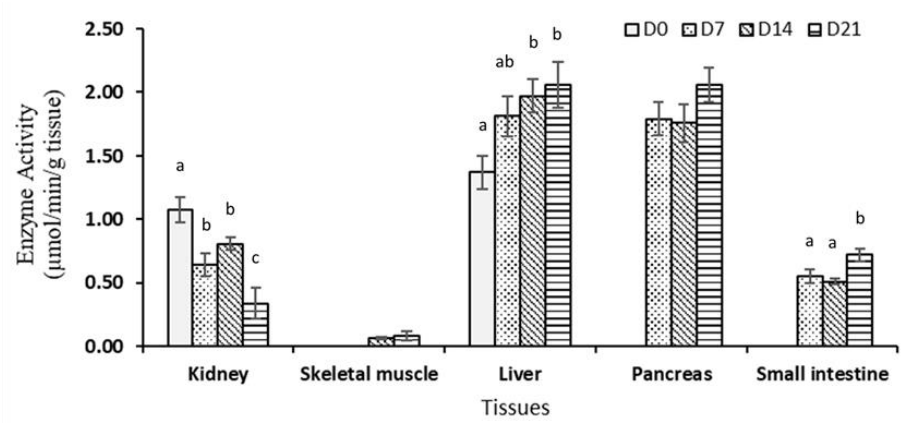
Figures 2.3 to 2.4 illustrate the enzymatic activities of HOA and AGT play roles in the metabolism of various amino acids to produce pyruvate, which is then oxidized via the Krebs cycle for energy (ATP) (Wang et al. 2014c). As shown in Figure 2.3, HOA activity was great from days 7 to 21 of age in the pancreas (no enzymatic activity on day 0), but enzymatic activity decreased moderately in the kidney with increasing age of the piglets. The activity of AGT increased in the liver during the first 3 weeks of life, decreased in the

kidney, and increased markedly by three-fold in both the pancreas and small intestine by postnatal day 21.



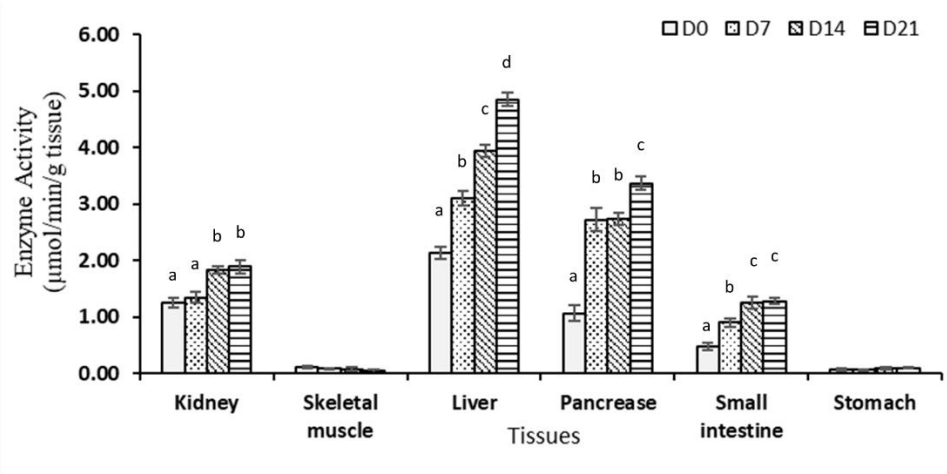
	Proline oxidase (POX) (µmole/min/g tissue)			
	Day 0	Day 7	Day 14	Day 21
Heart	-----	-----	-----	0.75 ± 0.09
Kidney	5.27 ± 0.28^{a}	3.12 ± 0.11^{b}	3.04 ± 0.15^{b}	2.13 ± 0.12^{c}
Skeletal Muscle	-----	0.86 ± 0.07^{a}	1.05 ± 0.05^{b}	1.22 ± 0.03^{c}
Liver	4.88 ± 0.15^{a}	2.55 ± 0.10^{b}	2.89 ± 0.13^{b}	2.10 ± 0.07^{c}
Pancreas	-----	2.22 ± 0.16^{a}	5.70 ± 0.24^{b}	7.13 ± 0.15^{c}
Small Intestine	1.01 ± 0.09^{a}	2.16 ± 0.06^{b}	3.50 ± 0.26^{c}	5.99 ± 0.18^{d}
Stomach	-----	-----	-----	0.68 ± 0.04

Figure 2.2. The enzymatic activity of proline oxidase (POX) in tissues from piglets at different days of age (days 0, 7, 14 and 21). The results indicated that the liver and kidney had the greatest proline oxidase activity at birth. Values are expressed as means ± SEM, n = 6 per time point. a-b means in a row without a common superscript letter differ ($P < 0.05$) as analyzed by one-way ANOVA, and intergroup comparisons were made by Student–Newman–Keuls (SNK).



	4-hydroxy-2-oxoglutarate aldolase (HOA) (µmole/min/g tissue)			
	Day 0	Day 7	Day 14	Day 21
Heart	-----	-----	-----	-----
Kidney	1.10 ± 0.10^{a}	0.65 ± 0.09^{b}	0.81 ± 0.05^{b}	0.34 ± 0.05^{c}
Skeletal Muscle	-----	-----	0.06 ± 0.01	0.08 ± 0.02
Liver	1.37 ± 0.13^{a}	1.81 ± 0.16^{ab}	1.97 ± 0.13^{b}	2.06 ± 0.18^{b}
Pancreas	-----	1.79 ± 0.13	1.76 ± 0.15	2.06 ± 0.14
Small Intestine	-----	0.55 ± 0.05^{a}	0.51 ± 0.02^{a}	0.72 ± 0.05^{b}
Stomach	-----	-----	-----	0.39 ± 0.04

Figure 2.3. The enzymatic activity for 4-hydroxy-2-oxoglutarate aldolase (HOA) in tissues from piglets on different ages (days 0, 7, 14 and 21). Values are expressed as means ± SEM, n = 6 per time point. a-b means in a row without a common superscript letter differ ($P < 0.05$) as analyzed by one-way ANOVA and the Student–Newman–Keuls (SNK) multiple comparison test.



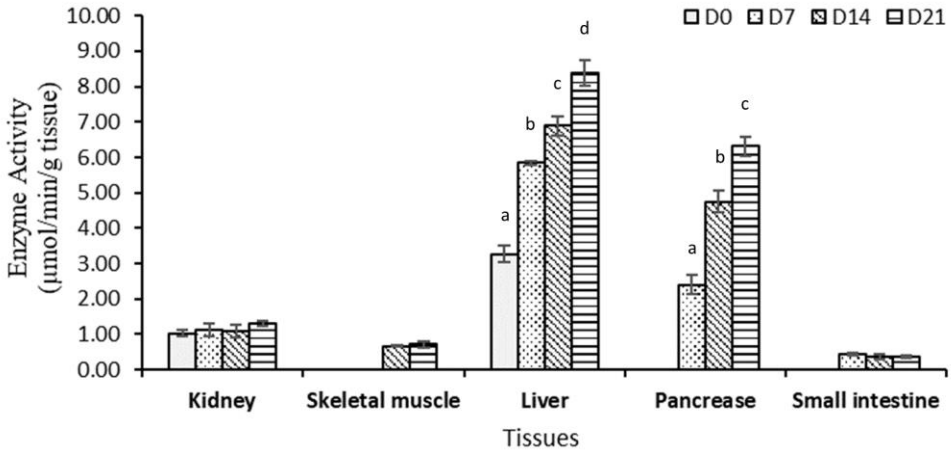
	Alanine:glyoxlate transaminase (AGT) (µmole/min/g tissue)			
	Day 0	Day 7	Day 14	Day 21
Heart	-----	-----	-----	-----
Kidney	1.25 ± 0.09^{a}	1.35 ± 0.10^{a}	1.84 ± 0.07^{b}	1.89 ± 0.12^{b}
Skeletal Muscle	0.12 ± 0.02	0.18 ± 0.02	0.18 ± 0.03	0.14 ± 0.01
Liver	2.14 ± 0.10^{a}	3.10 ± 0.13^{b}	3.95 ± 0.10^{c}	4.85 ± 0.12^{d}
Pancreas	1.07 ± 0.15^{a}	2.73 ± 0.20^{b}	2.75 ± 0.11^{b}	3.37 ± 0.12^{c}
Small Intestine	0.48 ± 0.06^{a}	0.91 ± 0.07^{b}	1.25 ± 0.11^{c}	1.28 ± 0.05^{c}
Stomach	0.07 ± 0.02	0.06 ± 0.01	0.09 ± 0.02	0.10 ± 0.02

Figure 2.4. The enzymatic activity for alanine:glyoxylate transaminase (AGT) in tissues from piglets on different days of age (days 0, 7, 14 and 21). Values are expressed as means ± SEM, n = 6 per time point. a-b means in a row without a common superscript letter differ ($P < 0.05$) as analyzed by one-way ANOVA and the Student–Newman–Keuls (SNK) multiple comparison test.

Enzymatic activities of serine-glycine and threonine–glycine pathways

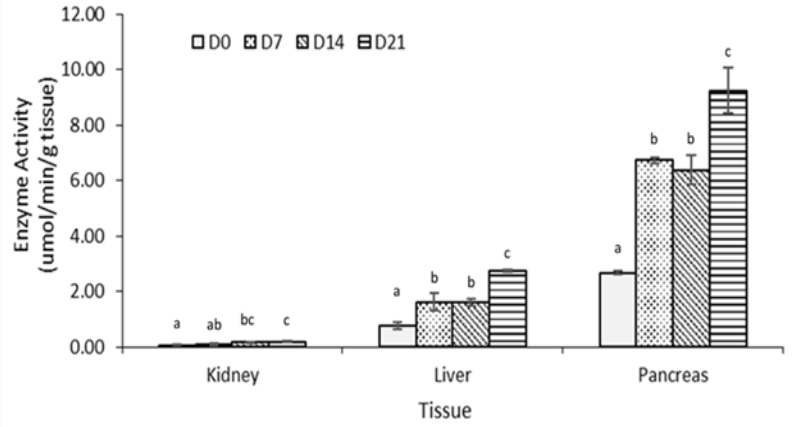
For comparing the capacity of the hydroxyproline-glycine pathway to synthesize glycine with that of the serine-glycine and threonine-glycine pathways, the activities of SHMT and TDH in tissues were determined. The liver and pancreas had the highest enzymatic activities among tissues between days 0 and 21 of age. The activity of SHMT in those two organs was lower at birth than at day 21 (Figure 2.5). Other organs had lower SHMT activity than the liver and kidneys. TDH activity was not detected in the heart, skeletal muscle, stomach, and small intestine of piglets at any age studied. As shown in

Figure 2.6, TDH activity in the pancreas, liver, and kidneys increased as the piglets grew ($P < 0.05$).



	Serine hydroxymethyltransferase (SHMT) (µmole/min/g tissue)			
	Day 0	Day 7	Day 14	Day 21
Heart	-----	-----	-----	-----
Kidney	1.02 ± 0.09	1.13 ± 0.19	1.08 ± 0.18	1.29 ± 0.07
Skeletal Muscle	-----	-----	0.67 ± 0.03	0.71 ± 0.09
Liver	3.26 ± 0.24^{a}	5.34 ± 0.06^{b}	6.89 ± 0.28^{c}	8.38 ± 0.37^{d}
Pancreas	-----	2.39 ± 0.27^{a}	4.75 ± 0.32^{b}	6.31 ± 0.26^{c}
Small Intestine	-----	0.44 ± 0.05	0.35 ± 0.08	0.36 ± 0.03
Stomach	-----	-----	-----	1.29 ± 0.26

Figure 2.5. The enzymatic activity for serine hydroxymethyltransferase (SHMT) in tissues from piglets on different days of age (days 0, 7, 14 and 21). Those results indicated that liver and pancreas are the main sites for serine-glycine conversion after day 14 of age. Values are expressed as means ± SEM, n = 6 per time point. a-b means in a row without a common superscript letter differ ($P < 0.05$) as analyzed by one-way ANOVA and the Student–Newman–Keuls (SNK) multiple comparison test.



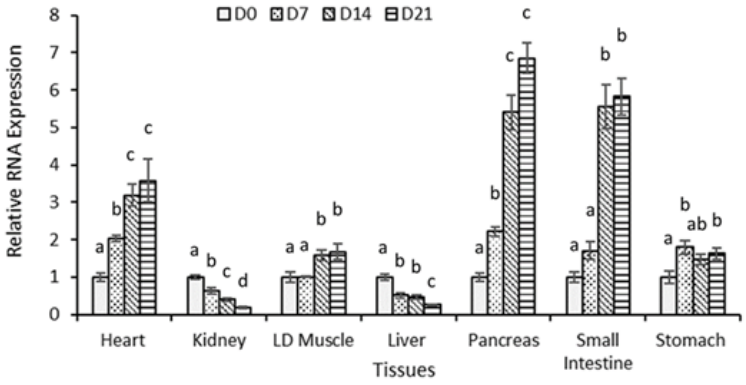
	Threonine dehydrogenase (TDH) (μmole/min/g tissue)			
	Day 1	Day 7	Day 14	Day 21
Kidney	0.03 ± 0.03^{a}	0.09 ± 0.04^{ab}	0.16 ± 0.02^{bc}	0.19 ± 0.01^{c}
Liver	0.77 ± 0.13^{a}	1.62 ± 0.33^{b}	1.59 ± 0.12^{b}	2.74 ± 0.03^{c}
Pancreas	2.68 ± 0.27^{a}	6.73 ± 0.11^{b}	6.37 ± 0.53^{b}	9.24 ± 0.82^{c}

Figure 2.6. The enzymatic activity of threonine dehydrogenase (TDH) in tissues from piglets on different days of age (days 0, 7, 14 and 21). Those results indicated that the conversion of threonine into glycine is limited in most tissues, and that liver and pancreas are the main sites for threonine-glycine conversion after day 14 age. Values are expressed as means ± SEM, n = 6 per time point. a-b means in a row without a common superscript letter differ ($P < 0.05$) as analyzed by one-way ANOVA and the Student–Newman–Keuls (SNK) multiple comparison test.

Expression of mRNA for enzyme proteins in tissues from piglets on different days of life

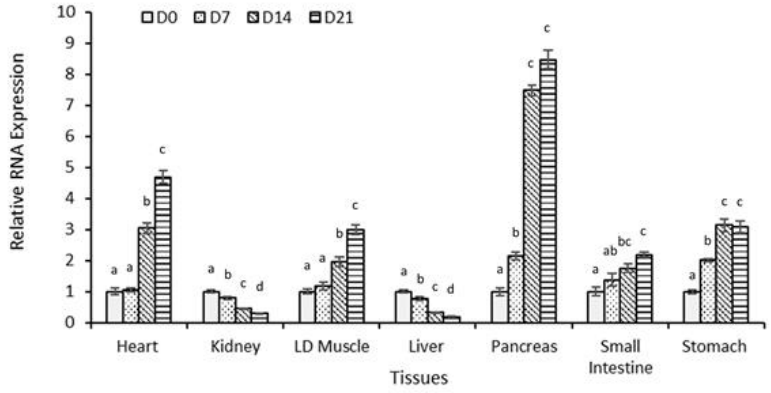
Quantitative real-time PCR was used for the analysis of genes encoding enzymes of the hydroxyproline-glycine pathway. Compared with results from the enzyme assays, expression of mRNAs for hydroxyproline oxidase (PRODH2) and proline oxidase (PRODH1) showed the same changes in the liver and kidneys, namely, high expression at birth that decreased with age. As shown in Figure 2.7 and 2.8, the expression of mRNAs for those two genes in the liver and kidneys were 5.6 and 5.8 times higher, respectively,

on day 0 than on day 21 ($P < 0.05$). At day 21 of age, the pancreas had the greatest expression of mRNAs for PRODH1 and PRODH2 among all of the tissues examined.



	mRNA expression for PRODH2			
	Day 0	Day 7	Day 14	Day 21
Heart	1.00 ± 0.12^{a}	2.03 ± 0.09^{b}	3.19 ± 0.29^{c}	3.58 ± 0.58^{c}
Kidney	1.00 ± 0.05^{a}	0.63 ± 0.09^{b}	0.40 ± 0.04^{c}	0.18 ± 0.03^{d}
Skeletal Muscle	1.00 ± 0.13^{a}	1.01 ± 0.03^{a}	1.59 ± 0.14^{b}	1.68 ± 0.21^{b}
Liver	1.00 ± 0.08^{a}	0.51 ± 0.06^{b}	0.47 ± 0.05^{b}	0.27 ± 0.01^{c}
Pancreas	1.00 ± 0.12^{a}	2.21 ± 0.13^{b}	5.41 ± 0.47^{c}	6.84 ± 0.41^{c}
Small Intestine	1.00 ± 0.15^{a}	1.71 ± 0.23^{a}	5.56 ± 0.59^{b}	5.82 ± 0.49^{b}
Stomach	1.00 ± 0.17^{a}	1.79 ± 0.18^{b}	1.48 ± 0.14^{ab}	1.63 ± 0.16^{b}

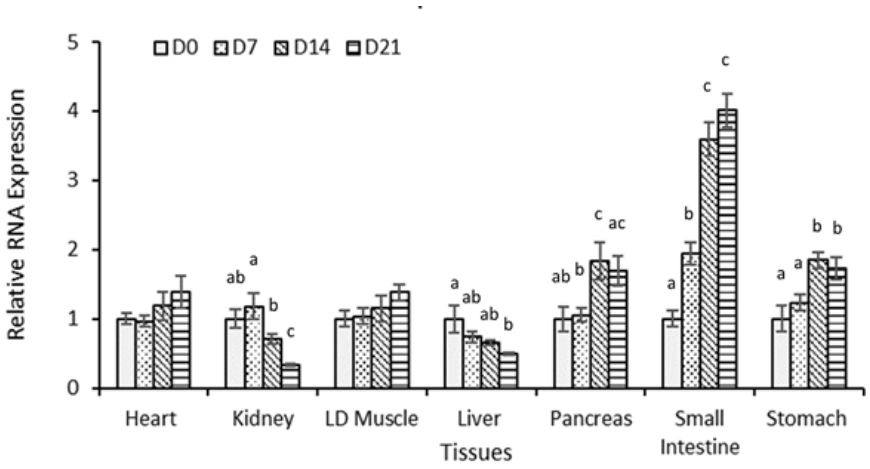
Figure 2.7. The expression of mRNA for hydroxyproline oxidase (gene name as PRODH2) in tissues from piglets on different days of age (days 0, 7, 14 and 21). Those results have the same tendency as OH-POX enzymatic activity with respect to age and growth. Values are expressed as means ± SEM, n = 6 per time point. a-b means in a row without a common superscript letter differ ($P < 0.05$) as analyzed by one-way ANOVA and the Student–Newman–Keuls (SNK) multiple comparison test.



	mRNA expression for PRODH			
	Day 0	Day 7	Day 14	Day 21
Heart	1.00 ± 0.11^{a}	1.07 ± 0.07^{a}	3.04 ± 0.17^{b}	4.67 ± 0.20^{c}
Kidney	1.00 ± 0.05^{a}	0.80 ± 0.04^{b}	0.45 ± 0.02^{c}	0.28 ± 0.02^{d}
Skeletal Muscle	1.00 ± 0.07^{a}	1.17 ± 0.13^{a}	1.96 ± 0.16^{b}	2.98 ± 0.15^{c}
Liver	1.00 ± 0.05^{a}	0.76 ± 0.06^{b}	0.32 ± 0.03^{c}	0.19 ± 0.01^{d}
Pancreas	1.00 ± 0.12^{a}	2.15 ± 0.13^{b}	7.48 ± 0.18^{c}	8.47 ± 0.29^{c}
Small Intestine	1.00 ± 0.17^{a}	1.37 ± 0.20^{ab}	1.75 ± 0.13^{bc}	2.19 ± 0.09^{c}
Stomach	1.00 ± 0.06^{a}	2.01 ± 0.05^{b}	3.14 ± 0.20^{c}	3.09 ± 0.18^{c}

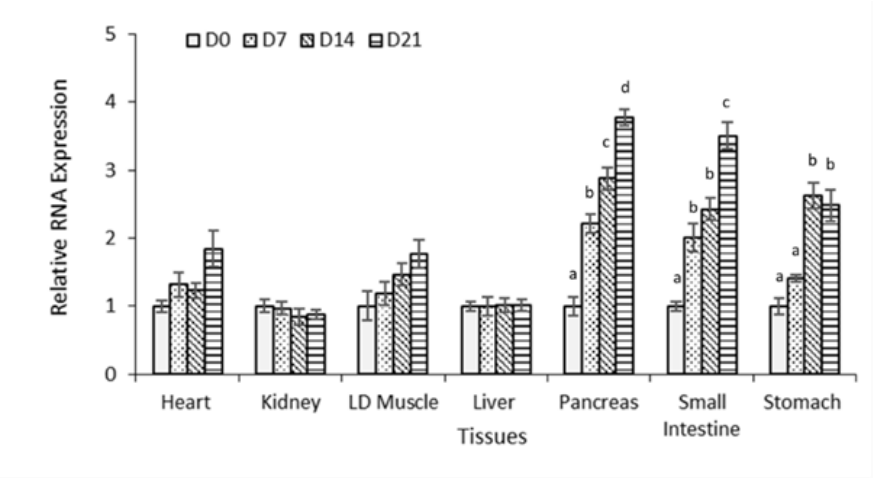
Figure 2.8. The expression of proline oxidase (gene name as PRODH1) mRNA in tissues from piglets on different days of age (days 0, 7, 14 and 21). Those results have the same tendency as POX enzymatic activity with respect to age and growth. Values are expressed as means ± SEM, n = 6 per time point. a-b means in a row without a common superscript letter differ ($P < 0.05$) as analyzed by one-way ANOVA and the Student–Newman–Keuls (SNK) multiple comparison test.

As the downstream enzymes in the hydroxyproline-glycine pathway, HOA and AGT convert the product of OH-POX into glycine (Valle et al. 1979; Wang et al. 2013a). Expression of mRNAs for those two genes increased with age in the pancreas and small intestine, but decreased in the liver (Figures 2.9 and 2.10). There were no appreciable changes in HOA and AGT in the liver or kidneys.



	mRNA expression for HOGA			
	Day 0	Day 7	Day 14	Day 21
Heart	1.00 ± 0.08	0.96 ± 0.08	1.19 ± 0.21	1.39 ± 0.22
Kidney	1.00 ± 0.05^{ab}	1.18 ± 0.19^{a}	0.71 ± 0.07^{b}	0.34 ± 0.02^{c}
Skeletal Muscle	1.00 ± 0.12	1.04 ± 0.12	1.15 ± 0.20	1.38 ± 0.11
Liver	1.00 ± 0.19^{a}	0.74 ± 0.08^{ab}	0.65 ± 0.03^{ab}	0.49 ± 0.02^{b}
Pancreas	1.00 ± 0.18^{ab}	1.05 ± 0.10^{b}	1.84 ± 0.27^{c}	1.69 ± 0.20^{ac}
Small Intestine	1.00 ± 0.14^{a}	1.94 ± 0.16^{b}	3.56 ± 0.24^{c}	4.01 ± 0.25^{c}
Stomach	1.00 ± 0.19^{a}	1.23 ± 0.11^{a}	1.85 ± 0.12^{b}	1.73 ± 0.16^{b}

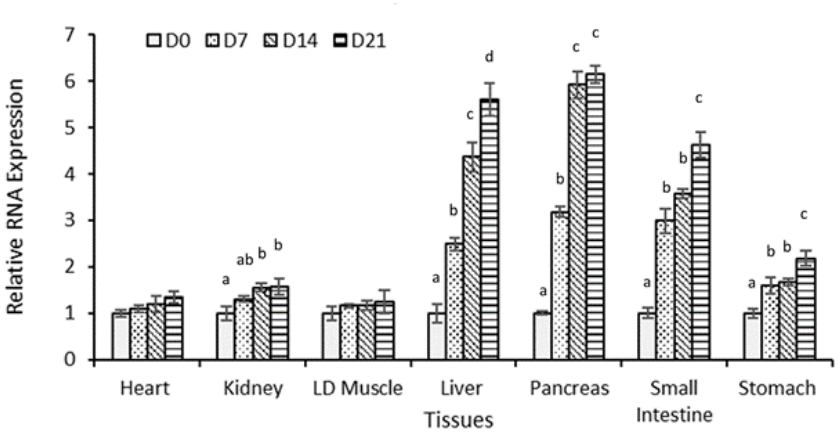
Figure 2.9. The expression of mRNA for 4-hydroxy-2-oxoglutarate aldolase (gene name as HOGA) in tissues from piglets on different days of age (days 0, 7, 14 and 21). Values are means ± SEM, n = 6 per time point. a-b means in a row without a common superscript letter differ ($P < 0.05$) as analyzed by one-way ANOVA and the Student–Newman–Keuls (SNK) multiple comparison test.



	mRNA expression for AGXT2			
	Day 0	Day 7	Day 14	Day 21
Heart	1.00 ± 0.09	1.31 ± 0.18	1.23 ± 0.12	1.45 ± 0.19
Kidney	1.00 ± 0.09	0.97 ± 0.10	0.85 ± 0.12	0.88 ± 0.06
Skeletal Muscle	1.00 ± 0.22	1.19 ± 0.16	1.46 ± 0.16	1.76 ± 0.21
Liver	1.00 ± 0.07	0.99 ± 0.13	1.02 ± 0.10	1.01 ± 0.09
Pancreas	1.00 ± 0.14^{a}	2.22 ± 0.14^{b}	2.88 ± 0.16^{c}	3.78 ± 0.11^{d}
Small Intestine	1.00 ± 0.07^{a}	2.01 ± 0.21^{b}	2.43 ± 0.17^{b}	3.51 ± 0.20^{c}
Stomach	1.00 ± 0.13^{a}	1.41 ± 0.05^{a}	2.62 ± 0.19^{b}	2.49 ± 0.22^{b}

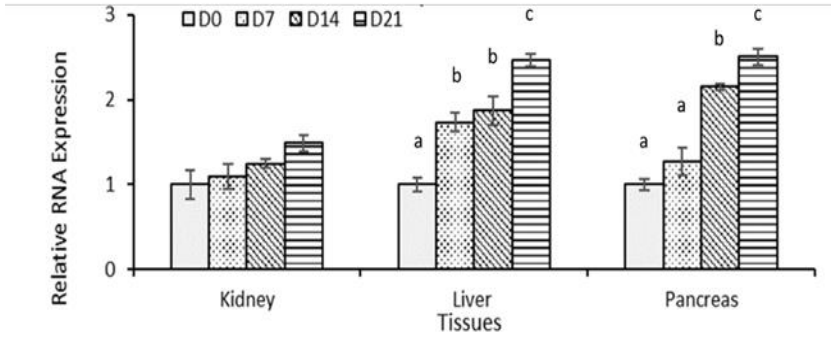
Figure 2.10. The expression of mRNA for alanine-glyoxylate transaminase (gene name as AGXT2) in tissues from piglets on different days of age (days 0, 7, 14 and 21). Values are expressed as means ± SEM, n = 6 per time point. a-b means in a row without a common superscript letter differ ($P < 0.05$) as analyzed by one-way ANOVA and the Student–Newman–Keuls (SNK) multiple comparison test.

The changes in expression of mRNAs for SHMT and TDH followed similar patterns to those for their enzymatic activities. In the liver and pancreas (Figures 2.11 and 2.12), the two genes had very limited expression at birth, but expression of their mRNAs increased with age ($P < 0.05$).



	mRNA expression for SHMT2			
	Day 0	Day 7	Day 14	Day 21
Heart	1.00 ± 0.08	1.09 ± 0.07	1.20 ± 0.17	1.35 ± 0.12
Kidney	1.00 ± 0.15^{a}	1.31 ± 0.06^{ab}	1.55 ± 0.09^{b}	1.57 ± 0.18^{b}
Skeletal Muscle	1.00 ± 0.15	1.16 ± 0.10	1.17 ± 0.10	1.25 ± 0.26
Liver53	1.00 ± 0.17^{a}	2.50 ± 0.14^{b}	4.37 ± 0.31^{c}	5.59 ± 0.35^{d}
Pancreas	1.00 ± 0.04^{a}	3.18 ± 0.12^{b}	5.92 ± 0.29^{c}	6.15 ± 0.19^{c}
Small Intestine	1.00 ± 0.12^{a}	2.99 ± 0.27^{b}	3.58 ± 0.09^{b}	4.63 ± 0.27^{c}
Stomach	1.00 ± 0.11^{a}	1.60 ± 0.18^{b}	1.67 ± 0.08^{b}	2.18 ± 0.16^{c}

Figure 2.11. Expression of mRNA for serine hydroxymethyltransferase (gene name as SHMT2) in tissues from piglets on different days of age (days 0, 7, 14 and 21). Values are expressed as means ± SEM, n = 6 per time point. a-b means in a row without a common superscript letter differ ($P < 0.05$) as analyzed by one-way ANOVA and the Student–Newman–Keuls (SNK) multiple comparison test.



	mRNA expression for TDH			
	Day 0	Day 7	Day 14	Day 21
Kidney	1.00 ± 0.17	1.10 ± 0.15	1.25 ± 0.05	1.49 ± 0.09
Liver	1.00 ± 0.08[a]	1.73 ± 0.11[b]	1.87 ± 0.16[b]	2.46 ± 0.07[c]
Pancreas	1.00 ± 0.06[a]	1.27 ± 0.16[a]	2.15 ± 0.04[b]	2.51 ± 0.09[c]

Figure 2.12. Expression of mRNA for threonine dehydrogenase (gene name as TDH) in tissue from piglets on different days of age (days 0, 7, 14 and 21). Values are expressed as means ± SEM, n = 6 per time point. a-b means in a row without a common superscript letter differ (P < 0.05) as analyzed by one-way ANOVA and the Student–Newman–Keuls (SNK) multiple comparison test.

Localization of OH-POX and POX proteins in pig tissues

In order to investigate the spatial and temporal expression of the OH-POX and POX proteins, we conducted immunohistochemical analyses of tissues. In the liver of piglets there were spatial changes in for both OH-POX and POX proteins with age (Figures 2.13 and 2.14). On day 0, OH-POX and POX proteins were specifically expressed in the periportal part of the classical liver lobule. On days 7 and 14, both proteins were present in the transitional part of the liver, and after postnatal day 21, the proteins were localized in the perivenous zone around the central vein in the liver.

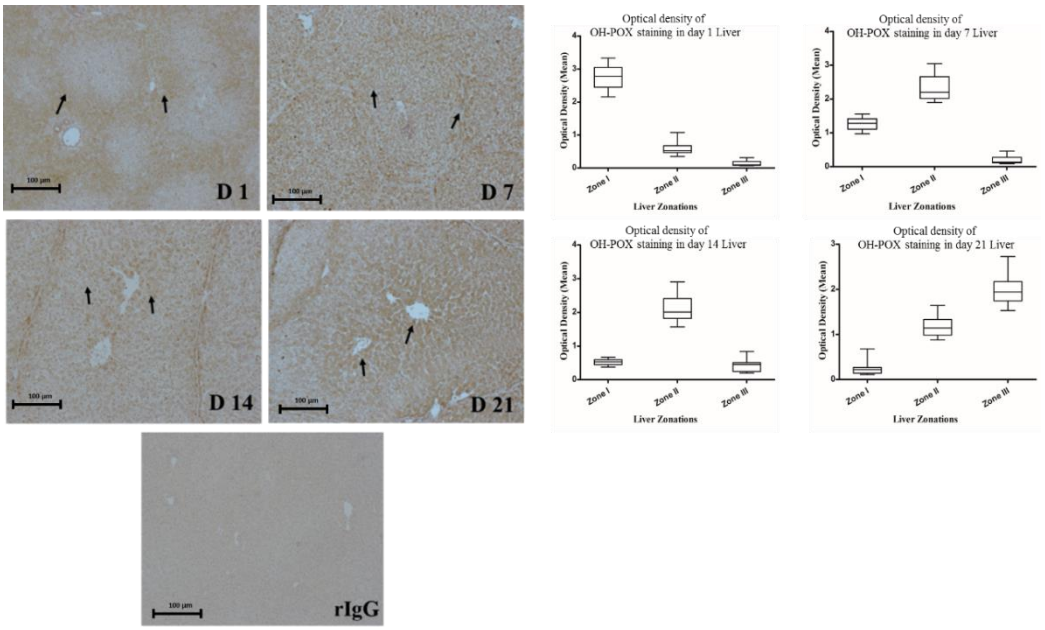


Figure 2.13. The localization and quantification, using optical density (OD), of hydroxyproline oxidase protein in the liver of piglets at different ages (day 0, 7, 14, and 21).

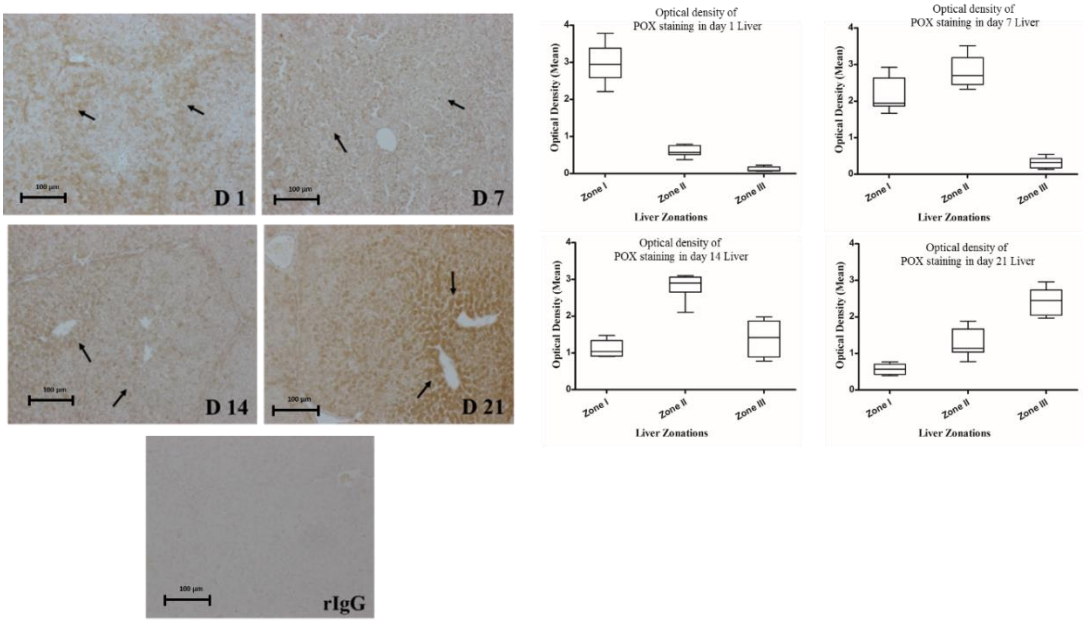


Figure 2.14. The localization and quantification, using optical density (OD), of proline oxidase protein in the liver of piglets at different ages (day 0, 7, 14, and 21).

The renal proximal tubule was the main site for OH-POX expression, and there was very limited expression in the distal tubules and glomeruli (Figure 2.15). In contrast, POX was expressed mainly in the distal tubules and glomeruli, but limited in the proximal tubule (Figure 2.16). Both OH-POX and POX proteins were highly expressed at birth and decreased to day 21, as observed for their enzymatic activities and mRNA transcripts.

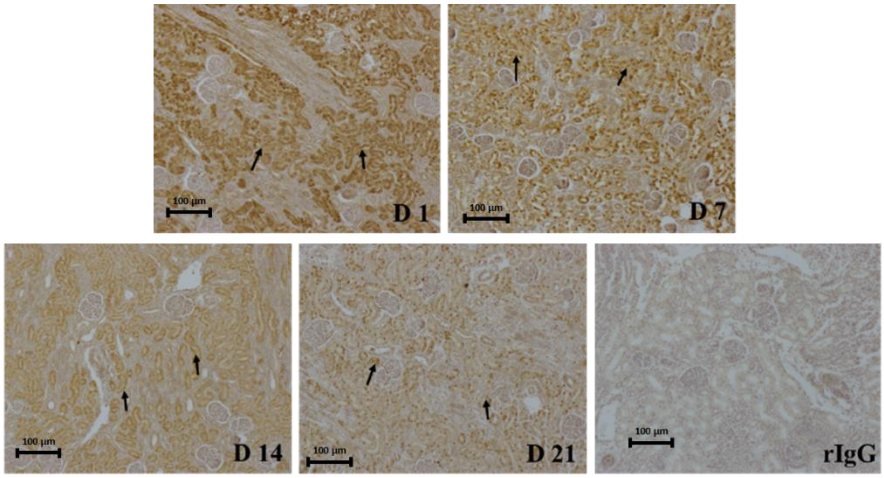


Figure 2.15. The localization of hydroxyproline oxidase protein in the kidney of piglets on postnatal days 0, 7, 14, and 21.

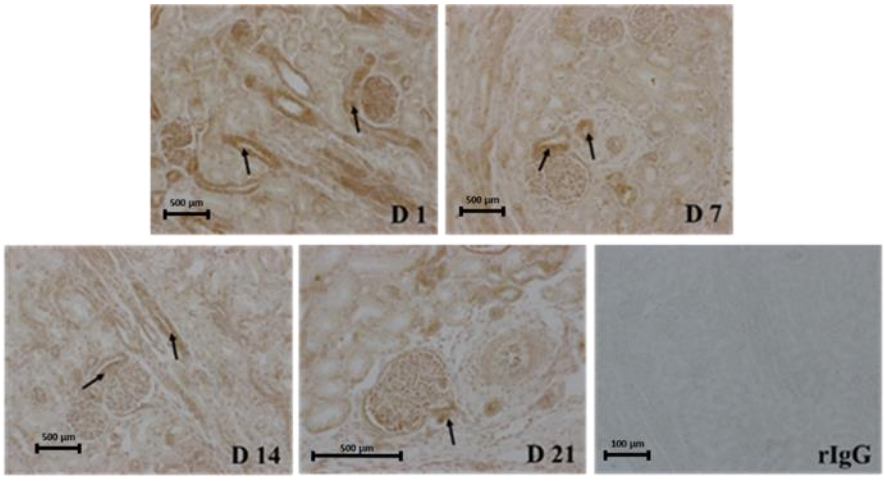


Figure 2.16. The localization of proline oxidase protein in the kidney of piglets on postnatal days 0, 7, 14, and 21.

As shown in Figures 2.17 and 2.18, the expression of OH-POX was low in the pancreas at day 0, but increased after 14 days of age. Expression of POX protein in the pancreas was not detected by IHC staining on postnatal day 0.

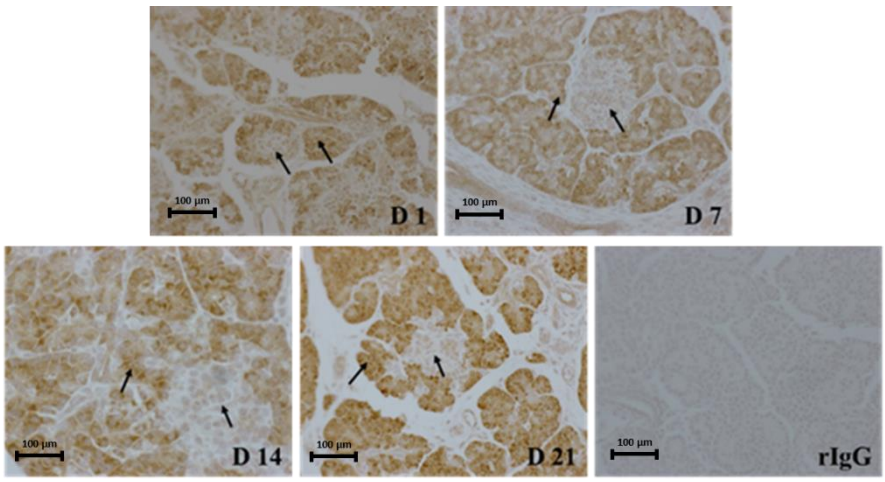


Figure 2.17. The protein of hydroxyproline oxidase protein in the pancreas on postnatal days 0, 7, 14, and 21.

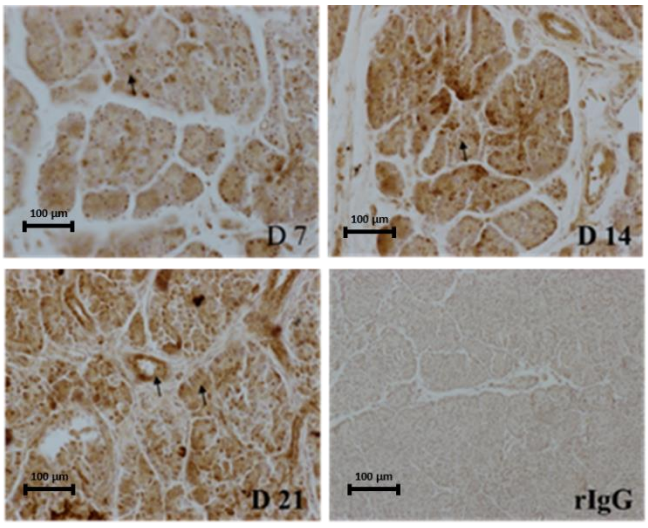


Figure 2.18. The localization of proline oxidase expression in the pancreas of piglets on postnatal days 7, 14, and 21.

For the small intestine (jejunum), the OH-POX and POX proteins were expressed in enterocytes and intestinal glands (crypts) (Figures 2.19 and 2.20). Epithelial cells and glands are the major sites for both nutrient absorption and protein secretion in the small intestine. The same results were found for the stomach (Figures 2.21), with epithelial cells and glands as the main sites for POX expression. The expression of OH-POX protein in the small intestine was not detected by IHC staining. Moreover, OH-POX protein was not detectable in the stomach, and POX protein was not detected in the stomach of piglets until postnatal day 21.

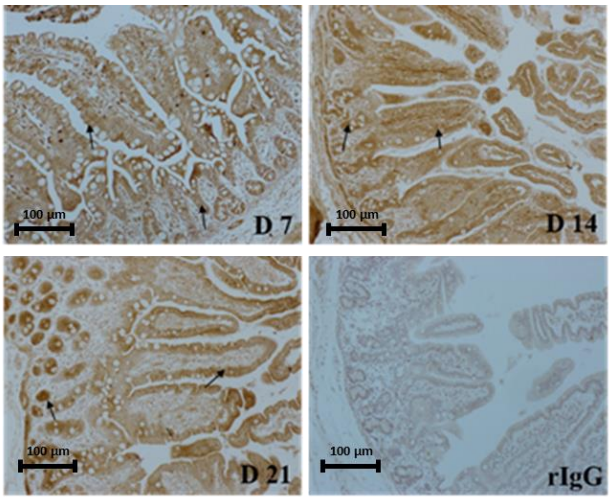


Figure 2.19. The localization of hydroxyproline oxidase protein in the small intestine of piglets on postnatal days 1, 7, 14, and 21.

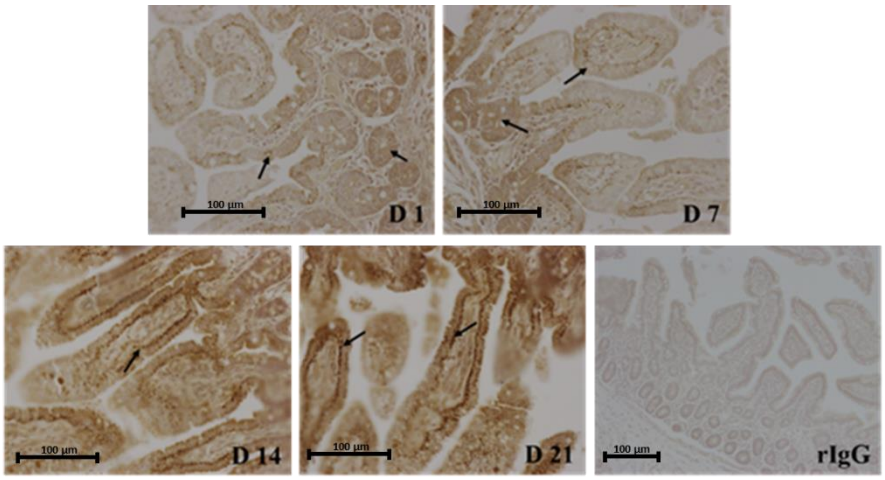


Figure 2.20. The localization of proline oxidase protein in the small intestine of piglets on postnatal days 1, 7, 14, and 21.

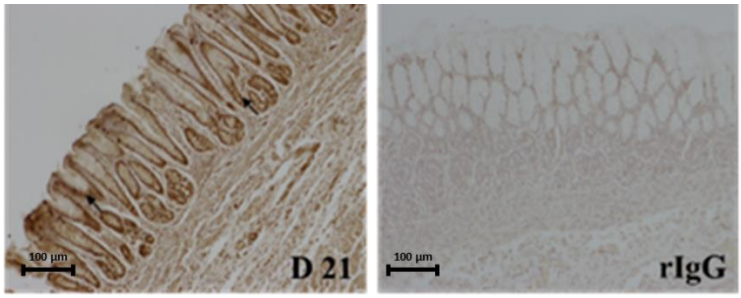


Figure 2.21. The localization of proline oxidase protein in the stomach of piglets on postnatal day 21.

Cardiac and skeletal muscles expressed OH-POX and POX at 21 days of age. IHC analyses detected both enzymes in the muscles between days 0 and 14 of life (Figures 2.22 and 2.23). Figure 2.23 clearly shows the difference between the cardiac muscle, skeletal muscle and smooth muscle (arterial vascular muscle). There was no staining for OH-POX and POX in smooth muscle, but a low level of staining was found in cardiac and skeletal muscles.

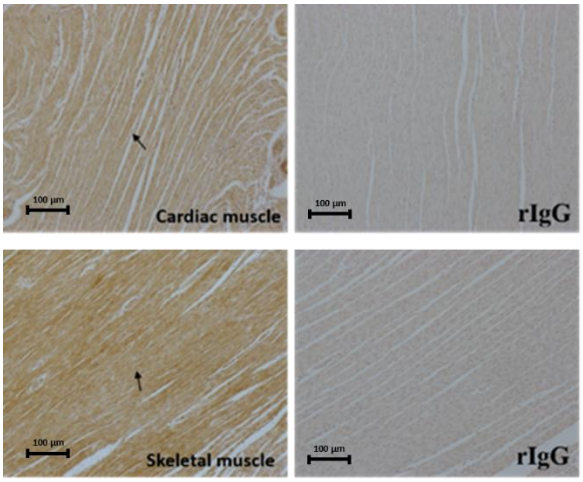


Figure 2.22. The localization of hydroxyproline oxidase protein in cardiac and skeletal muscle from piglets on postnatal day 21.

As shown for the liver (Figures 2.24 and 2.25), the bile duct in the liver of 21-day-old pigs expressed OH-POX and POX protein. These two proteins were also detected in the livers of piglets between postnatal days 0 and 14. Interestingly, both proteins were also present in epithelial cells of the gallbladder (Figures 2.24 and 2.25).

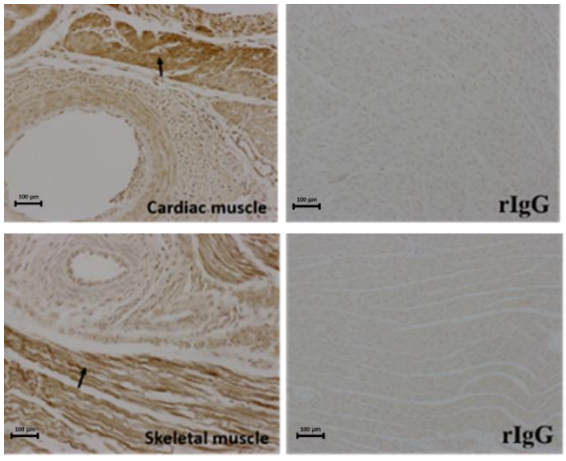


Figure 2.23. The localization of proline oxidase protein in cardiac and skeletal muscles on postnatal day 21.

Figure 2.24. The localization of hydroxyproline oxidase protein in both the bile duct, liver and gallbladder of piglets on postnatal day 21.

Figure 2.25. The localization of proline oxidase protein in both bile duct and liver (postnatal day 1) and gallbladder on postnatal day 21.

Glycine synthesis from tissues

As shown in Tables 2.2 to 2.5, concentrations of glycine in the liver and kidney, as well as culture medium, increased in a dose-dependent manner as concentrations of 4-hydroxyproline increased from 0 to 5 mM. This was also true for most of other tissues. Thus, the liver and kidney had a high ability to metabolize hydroxyproline into glycine in the first 2 to 3 weeks of postnatal life. However, the small intestine was a major organ for glycine synthesis from hydroxyproline in growing piglets. Muscle could convert hydroxyproline into glycine to some extent, but the heart, stomach, and gallbladder could not do so.

Table 2.2. Concentrations of glycine in incubation medium plus tissues from piglets on postnatal day 0 (nmol/mg tissue/2 h) *

	Concentrations of 4-Hydroxyproline in incubation medium of various tissues from neontal piglets						
	Background	0 mM	0.1 mM	0.2 mM	0.5 mM	2 mM	5 mM
Liver	1.36 ± 0.18^{a}	1.59 ± 0.15^{a}	3.32 ± 0.12^{b}	5.55 ± 0.47^{c}	9.97 ± 0.58^{d}	12.1 ± 0.57^{e}	16.3 ± 0.78^{f}
Pancreas	1.61 ± 0.20^{a}	3.68 ± 0.40^{b}	3.67 ± 0.57^{b}	3.15 ± 0.55^{b}	3.07 ± 0.13^{b}	3.03 ± 0.17^{b}	3.27 ± 0.21^{b}
Kidney	1.64 ± 0.10^{a}	2.05 ± 0.29^{a}	3.77 ± 0.04^{bc}	5.42 ± 0.15^{c}	9.09 ± 0.81^{d}	11.9 ± 0.83^{e}	14.2 ± 1.06^{f}
Small intestine	1.24 ± 0.03^{a}	2.10 ± 0.28^{b}	2.17 ± 0.35^{b}	2.15 ± 0.18^{b}	2.21 ± 0.28^{b}	2.15 ± 0.16^{b}	2.27 ± 0.29^{b}
Skeletal muscle	1.40 ± 0.05	1.82 ± 0.16	1.98 ± 0.17	1.48 ± 0.02	1.88 ± 0.17	1.89 ± 0.14	1.90 ± 0.12

* Values are as means ± SEM, n = 6 per time point. a-b means in a row without a common superscript letter differ ($P < 0.05$) as analyzed by one-way ANOVA and the Student–Newman–Keuls (SNK) multiple comparison test.

Table 2.3. Concentrations of glycine in incubation medium plus tissues from 7-day-old pigs (nmol/mg tissue/2 h) *

	Concentrations of 4-Hydroxyproline in incubation medium for tissues						
	Background	0 mM	0.1 mM	0.2 mM	0.5 mM	2 mM	5 mM
Liver	2.14 ± 0.11^{a}	2.45 ± 0.19^{a}	3.89 ± 0.44^{a}	6.13 ± 0.77^{b}	8.07 ± 0.58^{b}	12.9 ± 0.68^{c}	14.3 ± 1.83^{c}
Pancreas	2.36 ± 0.09^{a}	2.99 ± 0.04^{a}	4.34 ± 0.71^{ab}	4.74 ± 0.10^{bc}	6.17 ± 0.16^{c}	8.30 ± 0.24^{d}	8.82 ± 0.18^{d}
Kidney	2.64 ± 0.07^{a}	3.16 ± 0.38^{a}	3.97 ± 0.36^{a}	5.74 ± 0.69^{b}	8.09 ± 0.98^{c}	9.99 ± 0.72^{d}	12.4 ± 0.65^{e}
Small intestine	2.71 ± 0.16^{a}	4.66 ± 0.50^{ab}	4.88 ± 0.73^{ab}	5.96 ± 0.35^{b}	6.66 ± 1.11^{b}	7.20 ± 1.00^{b}	7.17 ± 0.75^{b}
Skeletal muscle	1.85 ± 0.12	1.98 ± 0.23	2.10 ± 0.12	2.16 ± 0.21	2.18 ± 0.29	2.15 ± 0.21	2.18 ± 0.04

* Values are means ± SEM, n = 6 per time point. a-b means in a row without a common superscript letter differ ($P < 0.05$) as analyzed by one-way ANOVA and the Student–Newman–Keuls (SNK) multiple comparison test.

Table 2.4. Concentrations of glycine in incubation medium plus tissues from 14-day-old pigs (nmol/mg tissue/2 h) *

	Concentrations of 4-Hydroxyproline in incubation medium						
	Background	0 mM	0.1 mM	0.2 mM	0.5 mM	2 mM	5 mM
Liver	1.53 ± 0.16^{a}	3.30 ± 0.52^{b}	4.47 ± 0.06^{bc}	5.44 ± 0.33^{c}	7.30 ± 0.78^{d}	11.2 ± 0.16^{e}	13.3 ± 1.15^{f}
Pancreas	1.93 ± 0.03^{a}	3.45 ± 0.88^{a}	7.00 ± 0.61^{b}	9.65 ± 1.18^{c}	13.5 ± 0.72^{d}	16.6 ± 1.14^{e}	16.2 ± 0.67^{e}
Kidney	2.74 ± 0.28^{a}	3.02 ± 0.57^{a}	4.02 ± 0.08^{a}	4.92 ± 0.91^{ab}	6.15 ± 0.37^{b}	8.62 ± 1.03^{c}	8.40 ± 0.20^{c}
Small intestine	2.96 ± 0.04^{a}	4.45 ± 0.14^{a}	5.48 ± 0.10^{a}	8.33 ± 0.25^{b}	12.4 ± 1.89^{c}	14.8 ± 0.30^{d}	15.7 ± 0.94^{d}
Skeletal muscle	1.11 ± 0.09^{a}	2.25 ± 0.64^{ab}	4.29 ± 1.03^{bc}	6.38 ± 1.49^{cd}	7.56 ± 1.19^{d}	10.8 ± 0.36^{e}	10.2 ± 0.12^{e}

* Values are as means ± SEM, n = 6 per time point. a-b means in a row without a common superscript letter differ ($P < 0.05$) as analyzed by one-way ANOVA and the Student–Newman–Keuls (SNK) multiple comparison test.

Table 2.5. Concentrations of glycine in incubation medium plus tissues from 21-day-old pigs (nmol/mg tissue/2 h) *

	Concentrations of 4-Hydroxyproline in incubation medium						
	Background	0 mM	0.1 mM	0.2 mM	0.5 mM	2 mM	5 mM
Liver	1.78 ± 0.28^{a}	3.15 ± 0.60^{ab}	4.61 ± 0.66^{b}	5.50 ± 0.11^{bc}	7.61 ± 0.54^{c}	10.9 ± 1.43^{d}	11.8 ± 1.16^{d}
Pancreas	0.98 ± 0.10^{a}	2.77 ± 0.51^{a}	5.72 ± 0.36^{b}	9.03 ± 0.93^{c}	12.7 ± 0.99^{d}	$13. 3 \pm 1.14^{d}$	16.1 ± 0.19^{e}
Kidney	2.15 ± 0.04^{a}	3.36 ± 0.29^{a}	4.95 ± 0.22^{b}	5.78 ± 0.83^{b}	6.52 ± 0.57^{bc}	8.02 ± 0.80^{cd}	8.00 ± 0.09^{d}
Small intestine	1.67 ± 0.23^{a}	3.54 ± 0.02^{ab}	5.59 ± 0.17^{bc}	7.96 ± 1.05^{c}	12.1 ± 1.81^{d}	15.4 ± 1.28^{e}	17.7 ± 0.94^{e}
Skeletal muscle	2.00 ± 0.07^{a}	3.37 ± 0.14^{ab}	4.58 ± 0.42^{bc}	5.90 ± 0.07^{c}	8.18 ± 0.40^{d}	9.59 ± 0.87^{e}	9.88 ± 0.69^{e}

* Values are as means ± SEM, n = 6 per time point. a-b means in a row without a common superscript letter differ ($P < 0.05$) as analyzed by one-way ANOVA and the Student–Newman–Keuls (SNK) multiple comparison test.

Discussion

Glycine, proline, and hydroxyproline are major constituents of extracellular matrix proteins, collagen and elastin (Wang et al. 2013a). The degradation of those proteins releases glycine-proline-hydroxyproline tripeptides into physiological fluids, such as blood and milk (Wu 2013a; Wu et al. 2011). Thus, this tripeptide is abundant in sow's milk (up to 10 mM) and plasma of piglets (up to 3 mM) (Wu et al. 2017). For comparison, glutamine, which is the most abundant amino acid in sow's milk, is present at 4 mM (Haynes et al. 2009), and glycine, which is the most abundant amino acid in plasma of piglets, is present at about 1 mM (Flynn et al. 2000). The unusual abundance of the Gly-Pro-Hyp tripeptide in milk and blood raised an important question about its nutritional and physiological significance.

The enzymatic activities of OH-POX, POX, HOA, AGT, SHMT, and TDH, which are involved in glycine synthesis, are summarized in Table 2.6. Hydroxyproline oxidase and POX play key roles in degrading hydroxyproline and proline, respectively. OH-POX and POX are highly expressed in the liver and kidneys of newborn pigs (Figures 2.1 & 2.2) and provide a biochemical basis for the utilization of hydroxyproline and proline by those tissues. Consistent with this observation is a previous report that after ^{14}C-labeled hydroxyproline and proline were administered into newborn pigs, the labeled amino acids rapidly appeared in their liver and kidneys to a much greater extent than in other organs (Bengtsson and Hakkarainen 1977). Thus, the liver and kidneys actively take up hydroxyproline and metabolize it to glycine in a concentration-dependent manner. This helps to explain high concentrations of glycine in plasma of piglets (Flynn et al. 2000).

Sufficient concentrations of glycine are needed to activate synthesis of muscle proteins and nucleotides (purines) (Sun et al. 2015).

The hydrolysis of the Gly-Pro-Hyp tripeptide releases hydroxyproline that is subsequently catabolized to glycine and yield 4 mol ATP/mol substrate (Li et al. 2017). Nutritional stress (e.g. glucose deficiency) increases POX expression, which increases proline degradation for production of energy (ATP) (Pandhare et al. 2009). Similar nutritional stress occurs in newborn pigs. Thus, the catabolism of hydroxyproline via OH-POX generates not only glycine, but also ATP to meet demands for the high metabolic rate of neonatal pigs.

Table 2.6. Comparison of enzymatic activities related to glycine synthesis

	Age of pigs			
Tissues	Day 0	Day 7	Day 14	Day 21
	Enzymatic activity of hydroxyproline oxidase			
Heart	**No**	**No**	**No**	+
Kidney	+++	++	++	+
Skeletal Muscle	**No**	**No**	++	++
Liver	++++	+++	+++	++
Pancreas	**No**	+	+++	++++
Small Intestine	**No**	**No**	++	+++
Stomach	**No**	**No**	**No**	**No**
	Enzymatic activity of proline oxidase			
Heart	**No**	**No**	**No**	+
Kidney	+++	+++	+++	+
Skeletal Muscle	**No**	**No**	+	+
Liver	+++	++	++	++
Pancreas	**No**	++	+++	++++
Small Intestine	+	++	++	+++
Stomach	**No**	**No**	**No**	**No**

Table 2.6. Continued

Tissues	Age of pigs			
	Day 0	Day 7	Day 14	Day 21
	Enzymatic activity of 4-hydroxy-2-oxoglutarate aldolase			
Heart	**No**	+	+	+
Kidney	++	++	++	+
Skeletal Muscle	**No**	**No**	**No**	**No**
Liver	++	++	++	++
Pancreas	**No**	+	++	++
Small Intestine	**No**	+	+	++
Stomach	**No**	**No**	**No**	**No**
	Enzymatic activity of alanine:glyoxylate transaminase			
Heart	**No**	**No**	**No**	**No**
Kidney	++	++	++	++
Skeletal Muscle	+	+	+	+
Liver	++	++	+++	+++
Pancreas	+	++	++	+++
Small Intestine	+	+	++	++
Stomach	+	+	+	+
	Enzymatic activity of serine hydroxymethyltransferase			
Heart	**No**	**No**	**No**	**No**
Kidney	++	++	++	++
Skeletal Muscle	**No**	**No**	+	+
Liver	+++	+++	++++	++++
Pancreas	**No**	++	++	+++
Small Intestine	**No**	+	+	+
Stomach	**No**	**No**	**No**	+
	Enzymatic activity of threonine dehydrogenase			
Heart	**No**	**No**	**No**	**No**
Kidney	+	+	++	++
Skeletal Muscle	**No**	**No**	**No**	**No**
Liver	+	++	++	+++
Pancreas	+	++	++	++++
Small Intestine	**No**	**No**	**No**	**No**
Stomach	**No**	**No**	**No**	**No**

The liver of piglets exhibits cell-specific expression of both OH-POX and POX. There are two different types of hepatocytes (periportal and perivenous) and three distinct zones in the liver (Figure 2.26). The periportal and perivenous hepatocytes have very different metabolic patterns (Gebhardt 1992; Haussinger et al. 1992; Jungermann 1986). The hepatic zonation includes periportal (zone I), transitional (zone II), and perivenous (zone III) zones. A change in hepatic protein localization of both OH-POX and POX may indicate a shift in their function between birth and weaning. The periportal hepatocyte contains abundant mitochondria and participates in amino acid synthesis and catabolism (Haussinger 1983; Haussinger et al. 1992; Jungermann 1986). However, the perivenous hepatocyte contains fewer mitochondria and they are involved in fatty acid metabolism (Jungermann 1986; Le Hir and Dubach 1980). The high abundance of OH-POX and POX proteins in the periportal hepatocytes at birth may indicate that those cells have a greater capacity to catabolize hydroxyproline and proline to generate glycine than the perivenous hepatocytes. As neonatal piglets grow, the small intestine expressed higher levels of OH-POX and POX (Figures 2.1 and 2.2) and becomes an active organ to degrading hydroxyproline and proline in milk to glycine and P5C, respectively. Thus, lesser amounts of these two dietary amino acids would enter the liver via the portal vein in 14- to 21-day-old pigs than in 0- to 7-day-old pigs. Those hydroxyproline and proline that enter the liver would largely bypass the periportal hepatocytes and be degraded to glycine in the perivenous hepatocytes where glycine oxidation is limited. This spatial expression of glycine-metabolic pathways is physiologically important to maximize the synthesis of glycine in the liver.

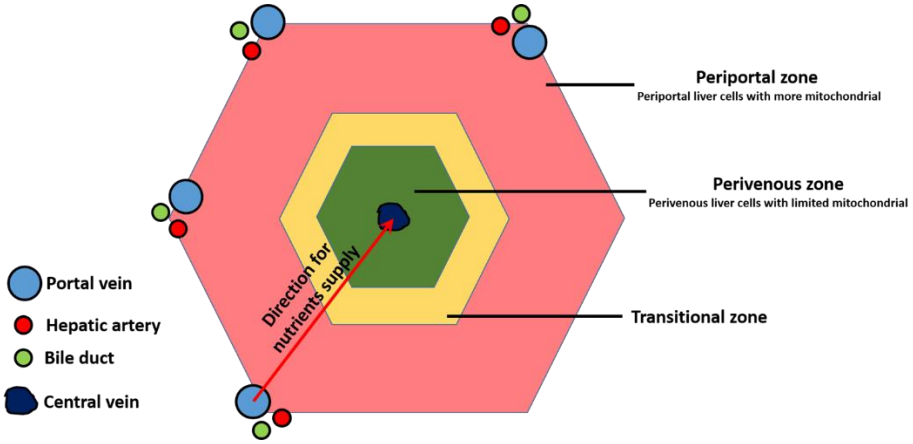


Figure 2.26. A schematic diagram of the liver with a classic lobule, zonation, and vessels. Pink area, periportal zone (zone I); yellow area, transitional zone (zone II): and green area, perivenous zone (zone III). Nutrients are supplied from the portal vein to periportal hepatocytes, and exit the liver through the central vein.

Any dietary hydroxyproline that escapes the liver and the hydroxyproline produced from the degradation of collagen and elastin in the connective tissue are taken up by the kidneys, skeletal muscle and possibly other tissues for glycine synthesis. In the kidneys, the proximal tubules of the cortex are the main site of OH-POX expression, hydroxyproline degradation, and glycine synthesis (Figure 2.15). Of interest, the proximal tubules of the kidney express argininosuccinate synthase and lyase for synthesis of arginine from citrulline, as well as arginine:glycine amidinotransferase that converts arginine and glycine into guanidinoacetate, the immediate precursor of creatine (Brosnan and Brosnan 2016). The colocalization of the enzymes for guanidinoacetate production in the same renal site can increase the efficiency of creatine synthesis. Likewise, the presence of glycine synthesis in skeletal muscle, which lacks enzymes for glycine degradation, can maximize the production of glycine locally to improve health and growth of piglets.

In conclusion, hydroxyproline is a major precursor for glycine synthesis in sow-reared pigs. OH-POX and POX, key enzymes for the degradation of hydroxyproline and proline as well as glycine synthesis, are highly expressed in the liver and kidneys of neonatal pigs. The pancreas, small intestine, and skeletal muscle of 14- to 21-day-old pigs synthesize glycine from hydroxyproline. The spatial and temporal changes in expression of glycine-synthetic enzymes maximize the net production of glycine to compensate for a severe deficiency of glycine in sow's milk, thereby supporting growth and development of piglets.

CHAPTER III

IMPAIRED ENDOGENOUS SYNTHESIS OF GLYCINE FROM HYDROXYPROLINE IN INTRA-UTERINE GROWTH RESTRICTED (IUGR) PIGLETS

Intrauterine growth restriction (IUGR) is one of the major problems in pig production, and there is a close association between IUGR and metabolic syndrome in later life. Previous evidence showed that the concentration of glycine in plasma of IUGR piglets is much lower than that in normal birth weight piglets. The present study was conducted to test the hypothesis that impairment of endogenous glycine synthesis contributes to glycine deficiency in IUGR piglets. At 0, 7, 14, and 21 days of age, six IUGR piglets and 6 normal birth-weight piglets were sacrificed, and samples of their tissues obtained for metabolic studies, as well as the determination of enzymatic activities, cellular localization and expression of mRNAs for glycine-synthetic enzymes. Our results indicated that: the enzymatic activities of hydroxyproline oxidase (OH-POX) and serine hydroxymethyl transferase (SHMT) were less in tissues of IUGR ($P < 0.05$) compared with normal piglets. The enzymatic activity of threonine dehydrogenase (TDH) did not differ between IUGR and normal piglets. Similar results were obtained for expression of mRNAs for those enzymes. Based on the results of immunohistochemistry (IHC), the abundance OH-POX protein was relatively low in the liver, small intestine, and skeletal muscle of IUGR piglets. These results indicate that endogenous synthesis of glycine is insufficient for maximal growth of sow-reared piglets.

Introduction

Glycine has critical physiological functions in nutrition, metabolism, and health. Beyond serving as a building block of protein (including collagen), glycine is required for the synthesis of heme, purines, creatine, glutathione, and bile salts (Amelio et al. 2014; Hall 1998; Jois et al. 1992; Li et al. 2016; Matilla et al. 2002; Mudd et al. 2007; Petrat et al. 2012). In addition, glycine participates in one-carbon unit metabolism, which is required for DNA synthesis and cell growth (Razak et al. 2017). Furthermore, glycine is an anti-oxidative and anti-inflammatory amino acid that modulates protein, glucose and lipid metabolism in cells and tissues (Amelio et al. 2014; Hall 1998; Jois et al. 1992; Li et al. 2016; Matilla et al. 2002; Mudd et al. 2007; Petrat et al. 2012; Razak et al. 2017; Sun et al. 2016a; Wang et al. 2014a; Wang et al. 2013a; Wang et al. 2014b; Weinberg et al. 2016; Wu et al. 2014). The amount of glycine in humans and animals is considerable. About 80% of dietary glycine is used for protein accretion, as glycine accounts for 11.5% of total amino acids in proteins of the whole body (Wang et al. 2013a; Wu et al. 2013; Yan and Sun 1997). Thus, a deficiency of glycine leads to growth restriction and oxidative stress in young pigs with a normal birth weight (Wang et al. 2014).

Intrauterine growth restriction (IUGR) is defined as impaired growth of a mammalian embryo/fetus or its organs, which may result from limited uterine capacity, insufficient nutrient supply or maternal endocrine disturbances during gestation (Wu et al. 2006). There is a higher occurrence of IUGR in polytocous than monotocous mammals, due to the limitations of the functional capacity of the placenta, uteroplacental transfer of nutrients and oxygen transport from mother to fetus (Bazer et al. 1969; Wang et al. 2017).

The IUGR animals are below either the 10th percentile or the population mean minus two standard deviations. Based on the large numbers of observations of birth weights of newborn piglets, a birth weight less than 1.1 kilograms meets the criterion of IUGR in modern breeds of piglets (Marsal 2002; Wu et al. 2004; Wu et al. 2006).

IUGR remains a major problem in the global swine industry. The pre-weaning survival rates decrease from 95 to 15% as piglet birth weights decrease from 1.80 to 0.61 kg (Wu et al. 2006). There is a close association between IUGR and onset of metabolic syndrome in later life (Wu et al. 2004). The concentration of glycine in the plasma of neonatal IUGR pigs is only about 50% of that in normal birth-weight piglets (Sun et al. 2015). IUGR offspring have increased risk for abnormal growth and development, hormonal imbalances, metabolic disorders, and inflammatory diseases (Barker and Clark 1997; Leon et al. 1998; Marsal 2002; Wu et al. 2004; Wu et al. 2006). This study tested the hypothesis that impaired synthesis of glycine from hydroxyproline contributed to a glycine deficiency in IUGR piglets. As reported in Chapter II, six IUGR piglets and six normal birth-weight piglets were sacrificed on postnatal days 0, 7, 14, and 21 and tissue samples were obtained for metabolic studies, as well as the determination of enzymatic activities, cell-specific localization and expression of mRNAs for glycine-synthetic enzymes.

Materials and methods

Pigs

The experimental protocol for this study was approved by the Texas A&M University Institutional Animal Care and Use Committee.

Piglets were offspring of Yorkshire x Landrace sows and Duroc x Hampshire boars maintained at the Texas A&M University Swine Center. Sows were nursing an average of 12 piglets at weaning. Throughout the lactation period, sows had free access to water and a corn-soybean meal based diet that met NRC requirements. Six IUGR and six normal birthweight piglets were selected randomly and sacrificed on postnatal days 0, 7, 14 or 21 when average body weights were 0.78 ± 0.18, 1.50 ± 0.45, 2.23 ± 0.70, and 3.44 ± 0.63 kg, respectively.

Collection of blood samples and analysis of amino acids.

On postnatal days 0, 7, 14 and 21, IUGR piglets were removed from sows for 1.5 h and jugular vein blood samples were withdrawn from each piglet into EDTA-containing tubes. The blood samples were centrifuged immediately at 10,000 rpm for 1 min, and plasma was stored at −80 °C until analyzed. Amino acids in plasma were analyzed by HPLC methods described previously (Hu et al. 2015; Wu et al. 1997; Wu and Knabe 1994; Wu and Meininger 2008).

Collection of tissues from IUGR piglets.

On postnatal days 0, 7, 14 and 21, after blood sampling, piglets were anesthetized with intramuscular injection of Telazol (10 mg/kg body weight) and then euthanized by intracardiac administration of saturated KCl. After the abdomen was opened, heart, liver, lung, stomach (with luminal contents removed), pancreas, spleen, jejunum (proximal half of the remaining small intestine, with luminal contents removed), kidney, gallbladder, skeletal muscle, skin, and brain were quickly isolated and weighted (Wang et al. 2014c).

Tissues were cut into small pieces, and a portion of the tissues was snap-frozen in liquid nitrogen and stored at −80°C while another portion of tissues was fixed in freshly prepared 4% (wt/vol) paraformaldehyde in PBS (pH 7.2) for 24 h and then transferred to 70% ethanol for 24 h. The fixed tissues were dehydrated through a graded series of alcohol to xylene (Wu et al. 1996) and embedded in Paraplast-Plus (Oxford Labware).

Analysis of enzymatic activities in tissues.

The homogenization of tissues and the determination of enzymatic activities were performed as described in Chapter II.

RNA isolation and quantitative Real-Time PCR analyses

Total RNA was isolated from tissues of piglets using Trizol (15596026; Invitrogen) according to Jobgen et al. (2009) (Jobgen et al. 2009), and the analysis of gene expression was performed using quantitative real-time RT-PCR, as described in Chapter II.

Immunohistochemical analyses

Immunohistochemical localization of OH-POX proteins in piglet tissues was performed as described in Chapter II.

Statistical analysis

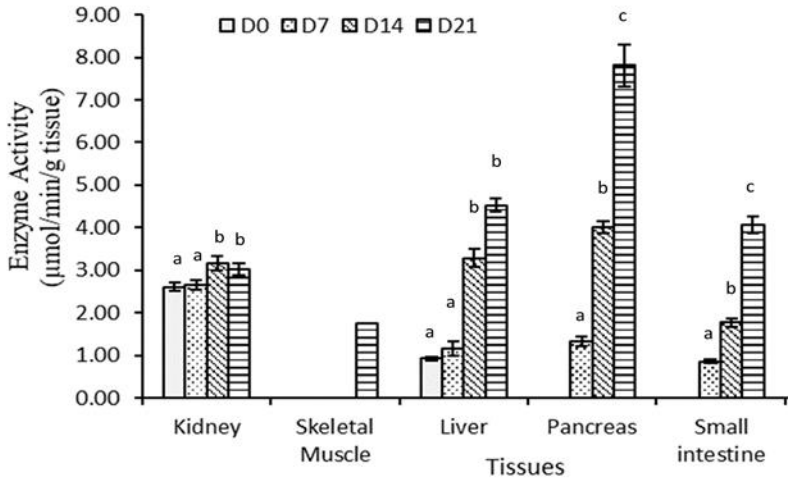
Results are expressed as means ± SEM. Statistical analyses of data were performed by one-way analysis of variance using the General Linear Models procedures (Assaad et al. 2014). Differences among treatment means were determined using the Student-Newman-

Keuls (SNK) multiple comparison method (Assaad et al. 2014). A probability value of ≤ 0.05 was taken to indicate statistical significance.

Results

Enzymatic activity of hydroxyproline oxidase

As discussed in Chapter II, hydroxyproline is the main substrate for glycine synthesis in neonatal piglets, and hydroxyproline oxidase (OH-POX) is the key and rate-controlling enzyme in the glycine-synthetic pathway (Lowry et al. 1985a; Phang et al. 1981; Ruiz-Torres and Kurten 1976; Takayama et al. 2003; Valle et al. 1979; Wu et al. 2011). Based on results of the previous studies (Chapter II), the heart, liver, stomach (with luminal contents being removed), pancreas, jejunum (proximal half of the remaining small intestine, with luminal contents being removed), kidneys, and skeletal muscle from IUGR piglets and piglets with normal birth weights on postnatal days 0, 7, 14 and 21 were analyzed.



	Hydroxyproline oxidase (OH-POX) (µmole/min/g tissue)			
	Day 0	Day 7	Day 14	Day 21
Kidney	2.61 ± 0.09^{a}	2.52 ± 0.11^{a}	3.17 ± 0.17^{b}	3.02 ± 0.14^{b}
Skeletal Muscle	-----	-----	-----	0.74 ± 0.10
Liver	0.93 ± 0.05^{a}	1.16 ± 0.18^{a}	3.29 ± 0.21^{b}	4.53 ± 0.16^{b}
Pancreas	-----	1.33 ± 0.12^{a}	4.01 ± 0.15^{b}	7.81 ± 0.49^{c}
Small Intestine	------	0.86 ± 0.05^{a}	1.77 ± 0.09^{b}	4.06 ± 0.20^{c}

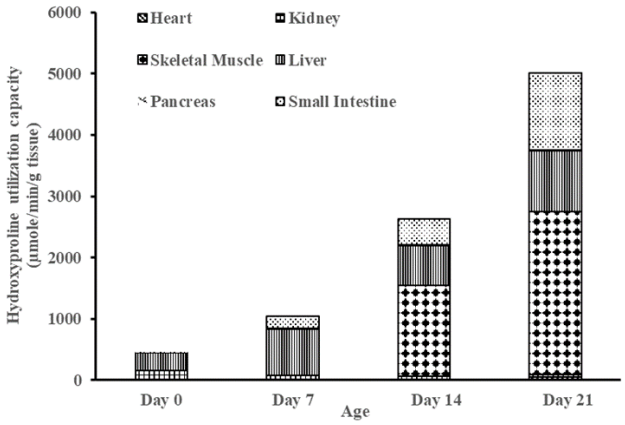
Figure 3.1. Enzymatic activity of hydroxyproline oxidase (OH-POX) in tissues from IUGR piglets on postnatal days 0, 7, 14 and 21. Values are expressed as means ± SEM, n = 6 per time point. Means in a row without a common superscript letter differ ($P < 0.05$) as analyzed by one-way ANOVA and the Student–Newman–Keuls (SNK) multiple comparison test.

Figure 3.1 shows the enzymatic activities of OH-POX in different tissues from piglets on postnatal days 0, 7, 14, and 21. OH-POX activity was not detected in cardiac muscle and stomach of piglets at any of the ages studied or skeletal muscle from 0- and 14-day-old IUGR pigs. Surprisingly, the enzymatic activity was low in the liver, kidneys, and pancreas of IUGR piglets on days 0 and 7 of age. Of note, OH-POX activity was not detected in the small intestine of IUGR piglets. The capacity of hydroxyproline utilization in different tissues of both normal and IUGR piglets are summarized in Figures 3.2 and 3.3. Specifically, IUGR piglets had only 6.8, 13.4, 6.6, and 68.8% of the capacity for

hydroxyproline utilization as that for normal birth-weight piglets on postnatal days 0, 7, 14, and 21, respectively. The contribution from the liver, small intestine, and skeletal muscle to glycine synthesis was limited in IUGR pigs.

	HYP utilization capacity in normal piglets (μmole/min)			
	Day 0	Day 7	Day 14	Day 21
Heart	0	0	0	45
Kidney	156	69.0	55.7	47.3
Skeletal Muscle	0	0	1485	2651
Liver	287	765	658	997
Pancreas	2.95	5.99	12.3	11.5
Small Intestine	0	198	421	1260
Sum	**446**	**1038**	**2632**	**5012**

Figure 3.2. Estimation of the capacity of different tissues from piglets of normal body weights to utilize hydroxyproline on postnatal days 0, 7, 14 and 21. Liver (high enzyme ability) and skeletal muscle (large mass, accounting for 40% body weight in neonatal piglets) were the main sites for hydroxyproline catabolism on days 0 and 7. The small intestine became an important site for glycine synthesis on days 14 and 21.



	HYP utilization capacity in IUGR piglets (µmole/min)			
	Day 0	Day 7	Day 14	Day 21
Kidney	14.4	30.5	48.6	75.5
Skeletal Muscle	0	0	0	2394
Liver	17.2	60.9	224	435
Pancreas	0	2.66	16.1	35.5
Small Intestine	0	45.5	151.2	512
Sum	**31.6**	**140**	**440**	**3452**

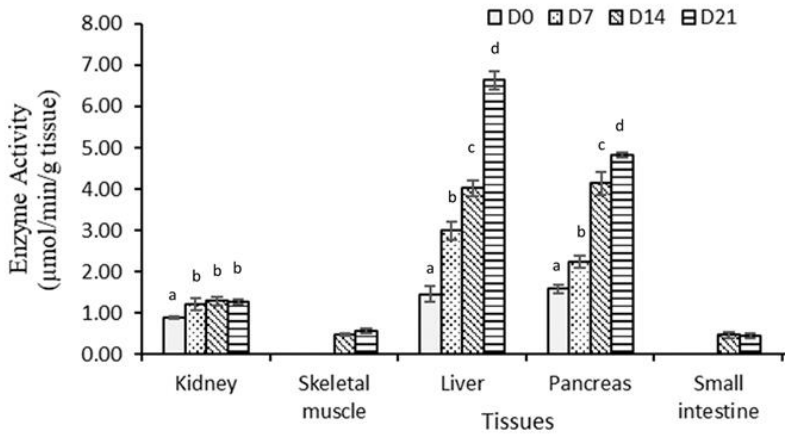
Figure 3.3. Estimation of the capacity of different tissues from IUGR piglets to utilize hydroxyproline on postnatal days 0,7,14 and 21. The utilization of hydroxyproline by IUGR piglets was low at birth compared with that for normal birth weight piglets, but increased with age.

Serine hydroxymethyl transferase activity

Serine is another source for glycine synthesis in neonatal pigs. The activities of serine hydroxymethyl transferase (SHMT) in tissues from different ages of IUGR piglets are shown in Figure 3.4. The SHMT activity increased in the liver, small intestine, and skeletal muscle from day 0 to day 21 of age. However, the enzymatic activity in the liver of IUGR piglets was very low compared with normal birth weight pigs. SHMT activities in the livers of IUGR piglets were only 27.8%, 56.0%, 58.5%, and 79.1% of those for normal

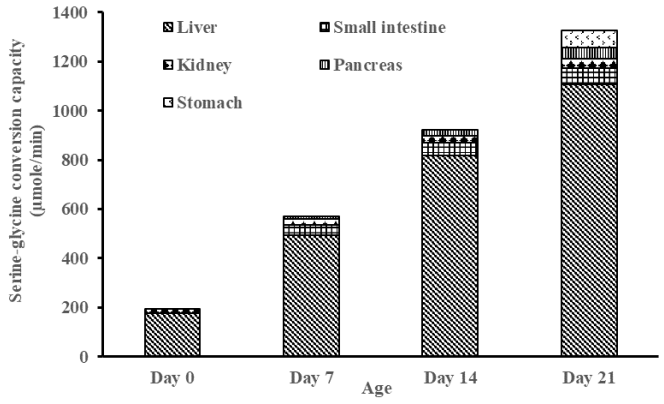
pigs on postnatal days 0, 7, 14, and 21, respectively. Because the liver is the main tissue for glycine synthesis via SHMT, this pathway was limited in neonatal IUGR piglets, especially in the first 2 weeks of life. The contributions of serine to glycine synthesis in different tissues of both normal and IUGR piglets are summarized as Figures 3.5 and 3.6.

Of note, IUGR piglets had only 16.3, 30.6, 40.0, and 65.9% of the serine-glycine conversion capacities as normal piglets at 0, 7, 14, and 21 days of age, respectively.



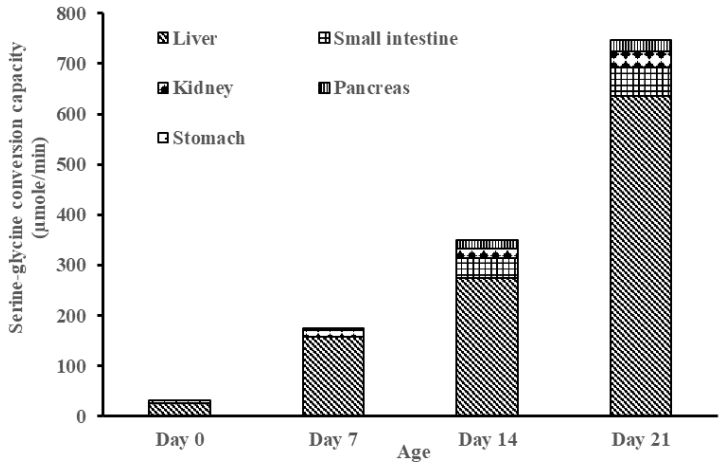
	Serine hydroxymethyl transferase (SHMT) (µmole/min/g tissue)			
	Day 0	Day 7	Day 14	Day 21
Kidney	0.90 ± 0.03^{a}	1.21 ± 0.14^{b}	1.29 ± 0.11^{b}	1.27 ± 0.05^{b}
Skeletal Muscle	-----	-----	0.49 ± 0.02	0.58 ± 0.06
Liver	1.46 ± 0.18^{a}	2.99 ± 0.20^{b}	4.03 ± 0.19^{c}	6.63 ± 0.22^{d}
Pancreas	-----	2.25 ± 0.14^{b}	4.14 ± 0.27^{c}	4.82 ± 0.06^{d}
Small Intestine	-----	-----	0.47 ± 0.08^{b}	0.45 ± 0.06^{b}

Figure 3.4. Enzymatic activity of serine hydroxymethyl transferase (SHMT) in tissues from IUGR piglets at different ages. Values are means ± SEM, n = 6 per time point. Means in a row without a common superscript letter differ ($P < 0.05$) as analyzed by one-way ANOVA and the Student–Newman–Keuls (SNK) multiple comparison test.



	Ser-Gly conversion capacity in normal piglets (µmole/min)			
	Day 0	Day 7	Day 14	Day 21
Kidney	15.8	23.2	28.0	40.3
Liver	177	492	819	1106
Pancreas	2.73	13.0	25.6	42.6
Small Intestine	28.9	43.4	50.0	66.6
Sum	**224**	**572**	**923**	**1255**

Figure 3.5. Estimation of the capacity for serine-glycine conversion in different tissues and at different ages for normal body weight piglets.



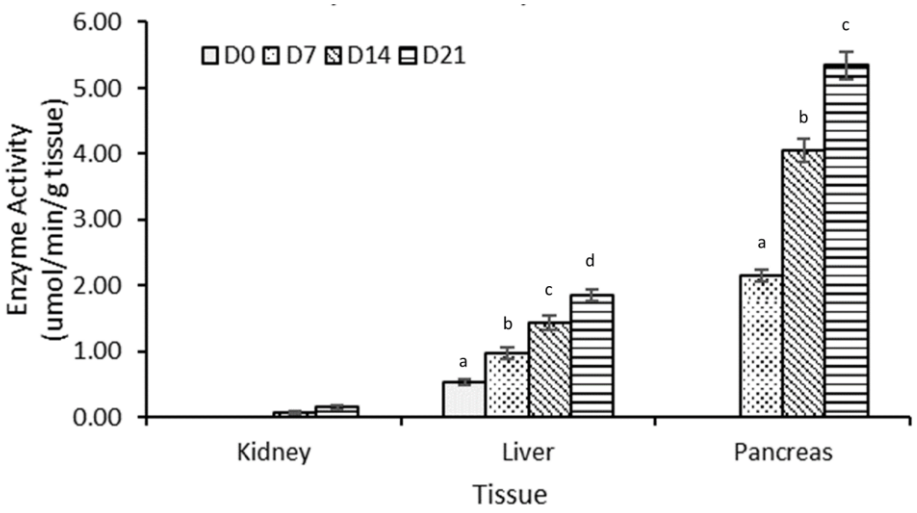
	Ser-Gly conversion capacity in IUGR piglets (µmole/min)			
	Day 0	Day 7	Day 14	Day 21
Kidney	4.95	13.9	19.4	31.8
Liver	26.3	157	274	636
Pancreas	1.59	4.50	16.6	21.7
Small Intestine	9.28	12.6	39.9	56.7
Stomach	0	9.78	27.0	44.7
Sum	42.1	198	377	791

Figure 3.6. Estimation of the capacities for serine-glycine conversion in different tissues of 0- to 21-day-old IUGR piglets.

Enzymatic activity of threonine dehydrogenase

The contribution of threonine to endogenous glycine synthesis was limited in neonatal piglets, as shown in Figure 3.7. TDH activities in the liver, kidney, and pancreas of IUGR piglets were much lower than those in normal piglets. In contrast to normal neonates, TDH expression in the kidney and pancreas was not detected in IUGR piglets at birth. The

activities in the liver and pancreas of IUGR piglets were, at most, only half of the values for normal birth weight piglets.



	Threonine dehydrogenase (TDH) (μmole/min/g tissue)			
	Day 0	Day 7	Day 14	Day 21
Kidney	-------	-------	0.07 ± 0.02	0.15 ± 0.03
Liver	0.53 ± 0.04[a]	0.98 ± 0.03[b]	1.43 ± 0.10[c]	1.85 ± 0.09[d]
Pancreas	-------	2.12 ± 0.09[a]	4.06 ± 0.18[b]	5.34 ± 0.20[c]

Figure 3.7. Enzymatic activity of threonine dehydrogenase (TDH) in tissues of IUGR piglets at different ages. Values are means ± SEM, n = 6 per time point. Means in a row without a common superscript letter differ ($P < 0.05$) as analyzed by one-way ANOVA and the Student–Newman–Keuls (SNK) multiple comparison test.

Expression of mRNAs for PRODH2, SHMT2, and TDH

Expression of mRNAs for PRODH2, SHMT2, and TDH are summarized in Table 3.1. The expression of PRODH2 (OH-POX) mRNA in the liver of IUGR pigs was low at birth, and increased with growth and age. At 21 days of age, no difference in hepatic expression of OH-POX was detected between IUGR and normal pigs. Similar results were obtained for kidneys and skeletal muscle. However, the expression of mRNA for PRODH2

remained lower in the small intestine and pancreas of 21-day-old IUGR piglets, compared with age-matched normal piglets ($P < 0.05$).

The liver is the main site for SHMT2 expression (Amelio et al. 2014; Schirch and Gross 1968; Shemin 1946). At 0 to 21 days of age, SHMT2 expression in the liver of IUGR pigs was much lower (only about 17%) than that in normal piglets. Of note, SHMT2 expression in the kidneys of IUGR piglets was also low between 0 and 21-days of age.

The contribution of TDH to glycine synthesis was limited in neonatal piglets. TDH activities in the kidney, liver, and pancreas were much lower for IUGR piglets than normal piglets.

Table 3.1. Relative expression of mRNAs for PRODH2, SHMT2, and TDH in different tissues from normal and IUGR piglets on postnatal days 0, 7, 14 and 21.

OH-POX	D 0		D 7		D 14		D21	
	Normal	IUGR	Normal	IUGR	Normal	IUGR	Normal	IUGR
Liver	1.00 ± 0.13^{a}	0.11 ± 0.01^{d}	0.63 ± 0.07^{b}	0.19 ± 0.02^{d}	0.59 ± 0.09^{bc}	0.28 ± 0.12^{d}	0.39 ± 0.04^{bd}	0.33 ± 0.03^{cd}
Kidney	1.00 ± 0.12^{a}	0.35 ± 0.03^{bc}	0.61 ± 0.03^{b}	0.30 ± 0.02^{bc}	0.49 ± 0.11^{bc}	0.36 ± 0.12^{bc}	0.22 ± 0.01^{c}	0.39 ± 0.04^{bc}
Pancreas	1.00 ± 0.10^{e}	0.32 ± 0.05^{e}	2.06 ± 0.14^{d}	0.90 ± 0.04^{e}	4.81 ± 0.36^{b}	2.97 ± 0.25^{c}	6.13 ± 0.45^{a}	4.85 ± 0.17^{b}
Small intestine	1.00 ± 0.08^{e}	0.09 ± 0.01^{f}	1.94 ± 0.22^{d}	0.38 ± 0.03^{f}	4.32 ± 0.22^{b}	3.29 ± 0.21^{c}	5.18 ± 0.28^{a}	4.20 ± 0.23^{b}
Skeletal muscle	1.00 ± 0.06^{cd}	0.24 ± 0.02^{e}	1.36 ± 0.19^{bc}	0.77 ± 0.08^{d}	1.64 ± 0.17^{ab}	1.06 ± 0.05^{cd}	1.89 ± 0.19^{a}	1.81 ± 0.15^{a}
				SHMT				
Liver	1.00 ± 0.04^{d}	0.17 ± 0.02^{e}	2.03 ± 0.18^{c}	0.68 ± 0.08^{d}	4.40 ± 0.11^{b}	1.05 ± 0.13^{d}	5.87 ± 0.26^{a}	4.73 ± 0.30^{b}
Kidney	1.00 ± 0.05^{cd}	0.88 ± 0.04^{d}	1.42 ± 0.16^{bc}	1.19 ± 0.16^{cd}	1.86 ± 0.16^{ab}	1.83 ± 0.17^{a}	1.91 ± 0.12^{ab}	1.89 ± 0.13^{ab}
Pancreas	1.00 ± 0.12^{d}	0.75 ± 0.07^{d}	3.06 ± 0.21^{c}	1.05 ± 0.12^{d}	5.17 ± 0.30^{b}	2.69 ± 0.27^{c}	5.76 ± 0.16^{a}	3.12 ± 0.19^{c}
Small intestine	1.00 ± 0.17^{d}	0.62 ± 0.02^{d}	3.11 ± 0.09^{b}	0.89 ± 0.06^{d}	5.45 ± 0.27^{a}	2.08 ± 0.22^{c}	5.90 ± 0.33^{a}	2.67 ± 0.21^{b}
Skeletal muscle	1.00 ± 0.09^{a}	0.07 ± 0.01^{d}	1.02 ± 0.07^{ab}	0.50 ± 0.03^{c}	1.11 ± 0.06^{a}	0.53 ± 0.03^{c}	1.09 ± 0.08^{a}	0.82 ± 0.06^{b}
				TDH				
Liver	1.00 ± 0.13^{d}	0.77 ± 0.05^{d}	1.59 ± 0.15^{bc}	1.14 ± 0.19^{cd}	1.91 ± 0.20^{b}	1.59 ± 0.14^{b}	2.51 ± 0.17^{a}	1.93 ± 0.05^{b}
Kidney	1.00 ± 0.15^{b}	0.12 ± 0.02^{d}	1.42 ± 0.10^{a}	0.13 ± 0.01^{d}	1.48 ± 0.13^{a}	0.33 ± 0.02^{cd}	1.63 ± 0.09^{a}	0.56 ± 0.02^{c}
Pancreas	1.00 ± 0.11^{c}	0.08 ± 0.01^{d}	1.51 ± 0.10^{b}	0.35 ± 0.02^{d}	2.03 ± 0.11^{a}	0.87 ± 0.10^{c}	2.30 ± 0.20^{a}	1.40 ± 0.13^{b}

Values are means ± SEM, n = 6 per time point. a-b means in a row without a common superscript letter differ ($P < 0.05$) as analyzed by one-way ANOVA and the Student–Newman–Keuls (SNK) multiple comparison test.

Localization and abundances of OH-POX protein in pig tissues

To investigate if there was a difference in localization and abundances of OH-POX protein, we performed immunohistochemical analyses. In the liver of IUGR piglets (Figure 3.8), the abundance of OH-POX protein was relatively low compared with normal piglets (Chapter II). Interestingly, the age-dependent change in the distribution of the OH-POX protein found in the different zones of the liver of normal piglets was not observed in the liver of IUGR piglets. The localization of the protein was disorganized in livers of IUGR piglets, compared with normal piglets. As noted in Chapter II, OH-POX protein was localized in the periportal zone at birth, appeared in the transitional zone at 7 days of age, and present in the perivenous zone at 21 days of age in normal piglets. In contrast, after day 14, the OH_POX protein seemed to be aggregated to the perivenous zone in the liver of IUGR piglets. These results suggest that the impairment of hydroxyproline catabolism in the liver may be caused by the limited expression of the OH-POX gene.

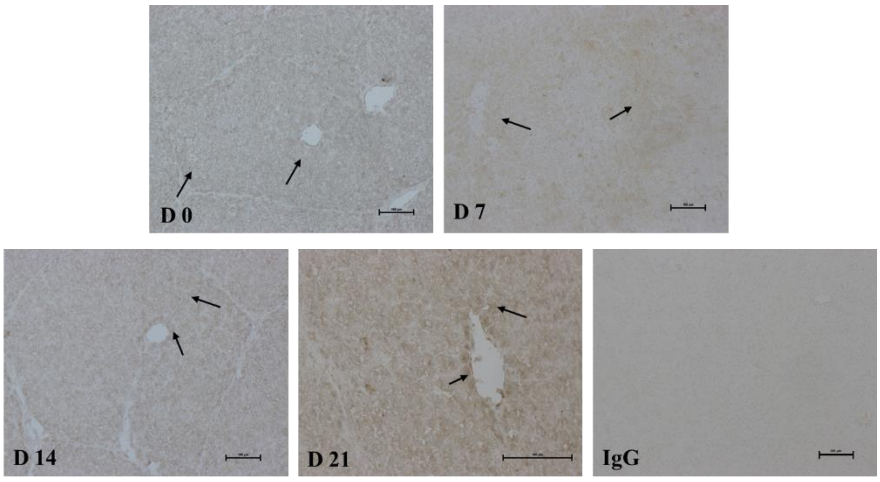


Figure 3.8. The abundance and localization of OH-POX protein in the liver of IUGR piglets at different ages.

The IHC results for the kidney of IUGR piglets showed that the OH-POX protein was localized in the proximal renal tubule and the abundance increased progressively from day 0 to day 21 of age. As shown in Figure 3.9, the renal OH-POX protein was expressed weakly on day 0, but more abundant after day 14 in IUGR piglets.

In the small intestine (jejunum), the OH-POX protein was expressed only in enterocytes, but not the intestinal glands (crypts) (Figure 3.10, day 0 of age). However, at days 14 and 21 of age, OH-POX protein was expressed in both enterocytes and the intestinal glands (crypts). Epithelial cells and glands are the major sites of both nutrient absorption and protein secretion in the small intestine. These results indicate increased expression and activities of OH-POX in the small intestine during the suckling period.

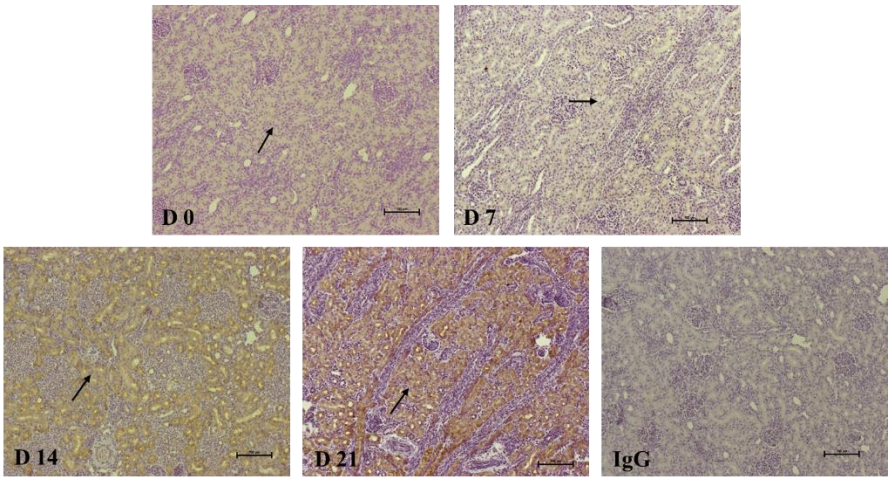


Figure 3.9. The abundance and localization of OH-POX protein in the kidney of IUGR piglets at different ages.

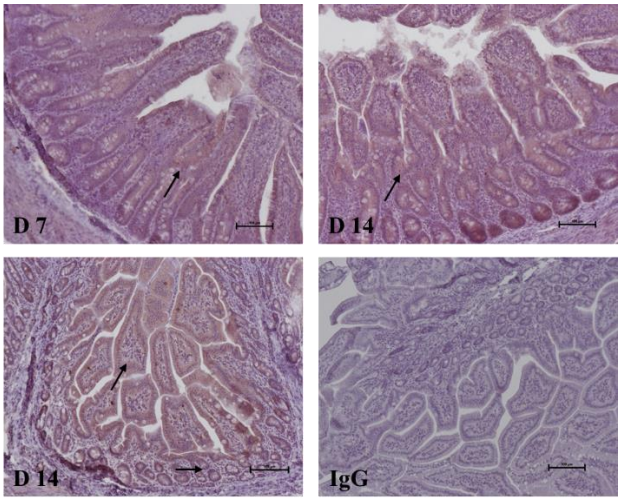


Figure 3.10. The expression and localization of OH-POX in the small intestine (jejunum) of IUGR piglets at different ages.

Unlike normal piglets, no OH-POX protein was detected in the pancreas, cardiac muscle, skeletal muscle, or stomach of IUGR piglets. Thus, tissue distribution of the OH-POX protein differed between normal and IUGR piglets.

Discussion

Unlike for other mammals, glycine is the most abundant amino acid in plasma of neonatal pigs (~1-1.2 mM), in comparison to 0.2-0.3 mM in plasma of other mammals (Wang et al. 2013a). However, as noted previously, concentrations of glycine in plasma of neonatal IUGR piglets is only about 0.6 mM. Considering the important physiological roles of glycine in purine synthesis, protein synthesis, one-carbon unit metabolism, cytoprotection (especially for liver cell and enterocytes in GI tract), anti-oxidation, and anti-inflammation (Jacob et al. 2003; Sun et al. 2016b; Wu 2013b), a deficiency in glycine may be one of the main factors contributing to high mortality rates and growth restriction in IUGR piglets (Wang et al. 2014a; Wu et al. 2014; Wu et al. 2013). At present, little is known about mechanisms responsible for glycine deficiency in IUGR neonates. OH-POX, serine hydroxymethyl transferase, and threonine dehydrogenase are key enzymes for the synthesis of glycine from hydroxyproline, serine, and threonine, respectively (Chao et al. 1953; Lowry et al. 1985a; Shemin 1946), respectively. Only by analyzing the expression of these enzymes, can we have an overall understanding of the capacities of tissues for endogenous glycine synthesis via different pathways in piglets. As discussed in Chapter II, the hydroxyproline-Gly pathway is primarily responsible for glycine synthesis in neonatal pigs. This pathway is seriously impaired in tissues of IUGR piglets. Similarly, the capacities for glycine synthesis via the threonine-glycine and the serine-glycine

pathways are much lower in the liver and kidneys of IUGR piglets, compared with normal piglets.

It should be borne in mind that rates of product formation in tissues are true indicators of the activities of their metabolic pathways, including the hydroxyproline-glycine pathway. This is because the measurement of enzymatic activity is performed under optimal conditions, such as saturated concentrations of substrates, which do not occur in tissues under physiological conditions. For this reason, the rate of glycine synthesis from hydroxyproline in tissues of IUGR and normal pigs must be determined. The localization of enzyme proteins in the liver is a determinant of their physiological functions. For normal piglets, OH-POX is expressed in the periportal zone of the liver in the first week of postnatal life to produce glycine (Chapter II). In contrast, this zonation of the OH-POX protein was not clearly observed in the liver of IUGR piglets. Rather, this protein was aggregated in the perivenous zone at 21 days of age. Because of the lack of the OH-POX protein in the periportal zone, the liver may contribute little or no glycine to IUGR piglets. In vivo measurement of glycine flux through the liver is required to test this hypothesis.

Besides the OH-POX-glycine pathway, the SHMT-glycine pathway is also impaired in IUGR pigs. This can be classified as “inborn errors of glycine synthesis” in pigs, which is a novel concept in amino acid metabolism. The pig, a polytocous animal, has a higher rate of IUGR occurrence than other livestock species. Limited uterine capacity results in an insufficient supply of nutrients and oxygen from mother to fetus. Increasing placental angiogenesis and growth is an effective means for enhancing fetal growth and development. We recently found that glycine promotes the transport of water and amino

acids by the porcine placenta (Hu et al. 2017, abstract). Thus, future studies are warranted to determine whether dietary supplementation with glycine may stimulate the glycine-synthetic pathways and improve embryonic and fetal development of mammals (including pigs).

In conclusion, results of the present study indicated that the enzymatic activities of OH-POX and SHMT were much lower in the liver and kidneys of IUGR piglets between days 0 and 14 of age, compared with normal pigs. The enzymatic activity of TDH in these two tissues did not differ between IUGR and normal piglets. Similar results were obtained for expression of mRNAs for those enzymes. The abundance of the OH-POX protein was low in the liver, small intestine, and skeletal muscle. Furthermore, there was no zonation of the OH-POX protein in the liver of IUGR piglets, possibly leading to a low net rate of glycine synthesis by the liver. These novel findings provide a biochemical basis to explain why the endogenous synthesis of glycine is insufficient for maximal growth of sow-reared IUGR piglets. This new foundational knowledge can be utilized to improve the growth, development, and survival of IUGR piglets worldwide.

CHAPTER IV

GLYCINE SUPPLEMENTATION ACTIVATES THE MTOR CELL SIGNALING IN SKELETAL MUSCLE AND ENHANCES THE GROWTH OF NEONATAL PIGLETS

This study was conducted to evaluate a role for glycine in the growth of both normal and low birth weight piglets reared by sows. Eighty newborn piglets (postnatal day 0), including 40 normal birth-weight piglets, body weight (BW) ≥ 1.10 kg, and 40 low birth-weight piglets (IUGR), BW < 1.10 kg, were allotted randomly into one of four treatment groups (10 normal birth-weight and 10 low birth-weight piglets in each group). Piglets received oral administration of either 0, 0.1, 0.2 or 0.4 g glycine per kg BW twice daily between 1 and 14 days of age. Alanine was used as the isonitrogenous control. The body weights of all piglets were recorded each week during the experiment. Two weeks after the initiation of glycine supplementation, blood and tissue samples were collected for biochemical assays. Results were analyzed statistically using one-way ANOVA. Compared with control piglets, oral administration of 0.2, 0.4 and 0.8 g glycine/kg BW per day enhanced ($P < 0.05$) concentrations of glycine and serine in plasma, as well as weight gain in piglets, with the dose of 0.4 g glycine/kg BW being the most effective. Consistent with its growth-promoting effect, glycine supplementation augmented the phosphorylation of mechanistic target of rapamycin (MTOR), eukaryotic initiation factor 4E binding protein 1 (4E-BP1) and p70S6 kinase (p70S6K) in skeletal muscle, compared with the control group. Collectively, oral administration of glycine activated the MTOR

signaling pathway in skeletal muscle and enhanced growth performance of newborn piglets, especially those with a low birth weight. The results also indicated that endogenous synthesis of glycine is inadequate to meet the metabolic needs of piglets with a low or normal birth weight.

Introduction

In both the fetus and the neonate, glycine has the greatest rate of deposition among all amino acids (Wu et al. 2014). Thus, dietary requirements of animals for glycine are particularly high during any phase of animal life (Hu et al. 2016; Wu 2009; Wu et al. 2014). This is consistent with multiple roles of glycine in cell signaling, metabolism, and function, including activation of the mechanistic target of rapamycin (MTOR) cell signaling pathway in skeletal muscle (Jackson 1991; Mudd et al. 2007; Wang et al. 2013a). Traditionally, glycine was classified as a "non-essential amino acid" for mammals, because it can be synthesized from serine (Shemin 1946), threonine (Chao et al. 1953), and choline (Soloway and Stetten 1953). However, growing evidence shows that the amount of endogenously synthesized glycine is insufficient to meet the metabolic requirements for humans, pigs, rodents, and birds (Jackson 1991; Wang et al. 2013a; Wang et al. 2014c; Wu and Knabe 1994). Therefore, glycine should be considered as a "conditionally essential amino acid" for animal growth, development, and health.

Intrauterine growth restriction (IUGR) is a major problem in swine production worldwide (Wu et al. 2006). Up to 25% of pigs are born with a body weight of less than 1.1 kg. Without effective nutritional management, neonatal pigs with IUGR grow at a

slower rate and have a lower feed efficiency and higher mortality than littermates with a normal birth weight (Ji et al. 2017). Thus, IUGR piglets are often culled on farms, and this represents huge economic losses to producers. Wang et al. (2014) reported that dietary supplementation with glycine improved the growth performance of milk-fed piglets with a normal birth weight. Whether this treatment can be applicable to IUGR piglets is unknown, because they may respond differently to the same nutrient (e.g., leucine or protein) than piglets with a normal birth weight (Sun et al. 2015). For example, doubling protein intake by 50% promotes muscle growth in piglets with a normal birth weight, but causes high rates of morbidity and mortality in piglets with IUGR (Jamin et al. 2010).

The objective of this study was to test the hypothesis that oral administration of glycine to sow-reared IUGR piglets could stimulate MTOR cell signaling and whole-body growth.

Materials and methods

Pigs

The experimental protocol of this study was approved by the Texas A&M University Institutional Animal Care and Use Committee. All animals were maintained at the Texas A&M University Swine Center.

Eighty newborn piglets were selected from 10 sows, and piglets were the offspring of Yorkshire x Landrace sows bred to Duroc boars. The average litter size of sows was 12 at birth. Throughout the lactation period, sows had free access to water and a corn-soybean meal-based diet that met the recommended NRC requirements (Table 4.1). Immediately after farrowing, all newborn piglets (day 0 of age) were weighted. Forty normal birth

weight piglets (normal, BW ≥ 1.1 kg), and 40 low birth weight piglets (IUGR, BW < 1.1 kg) were selected. Normal birth weight piglets or IUGR piglets were assigned randomly into one of four groups (10 piglets/group).

Experimental design

Beginning on the day of birth (day 0), immediately after nursing, piglets received oral administration of 0.1, 0.2, 0.4 g glycine/kg BW twice daily (in the morning and evening) for 14 days. L-alanine was used as the isonitrogenous control. Glycine or alanine was dissolved in 10 ml drinking water before gavaging. Piglets were weighed at days 0, 7 and 14 of age.

Blood sample collection and analysis of amino acids in plasma

On day 14 of the experiment, 6 piglets in each group were randomly selected and fasted for 1.5 h. Jugular vein blood samples (5 ml) were withdrawn from each animal into an EDTA-containing tube. The blood samples were centrifuged immediately at 10,000 rpm for 1 min, and the supernatant fluid (plasma) was stored at -80°C until analyzed. Amino acids in plasma were analyzed by HPLC methods, as previously described (Hu et al. 2015; Wu et al. 1997; Wu and Knabe 1994; Wu and Meininger 2008).

Table 4.1. Composition of the diet for lactating sows (as-fed basis)

Item	Diet[1], %
Ingredient	
Corn grain	57.50
Soybean meal, 44.5% CP	27.00
Cornstarch	2.0
Sugarcane molasses	3.85
Potassium chloride	0.10
Salt	0.35
Vitamin-mineral premix[2]	3.00
Vegetable oil	3.00
Dicalcium phosphate	2.50
Limestone	0.70
Chemical composition	
Dry matter, %	90.0
Metabolizable energy, Mcal/kg	3.32
Crude protein[3], %	17.5
Calcium, %	1.04
Available phosphorus, %	0.54
Total phosphorus, %	0.79

[1] This lactation diet was provided to sows ad libitum from farrowing.

[2]The vitamin premix provided the following per kg of complete diet (as-fed basis): 46.7 mg of Mn as manganous oxide; 75 mg of Fe as iron sulfate; 103.8 mg of Zn as zinc oxide; 9.5 mg of Cu as copper sulfate; 0.72 mg of I as ethylenediamine dihydroiodide; 0.23 mg of Se as sodium selenite; 7,556 IU of vitamin A as vitamin A acetate; 825 IU of vitamin D3; 61.9 IU of vitamin E; 4.4 IU of vitamin K as menadione sodium bisulfate; 54.9 µg of vitamin B12; 13.7 mg of riboflavin; 43.9 mg of D-pantothenic acid as calcium pantothenate; 54.9 mg of niacin; and 1,650 mg of choline as choline chloride.

Collection of tissues from piglets.

On day 14 of the experiment, after blood sampling, piglets were anesthetized with an intramuscular injection of Telazol (10 mg/kg BW) and then euthanized by intra-cardiac administration of saturated KCl. Skeletal muscle (*Latissimus dorsi* muscle) was quickly isolated and cut into small pieces which were frozen in liquid nitrogen, and stored at -80°C.

Western blot analysis

The frozen muscle tissue was ground to powder under liquid nitrogen and then homogenized in the lysis buffer containing 20 mM Tris-HCl (pH 7.4), 50 mM NaCl, 50 mM NaF, 50 mM of EDTA, 1% Triton X-100, 1x protease inhibitor cocktail, and 1x phosphatase inhibitor cocktail (Calbiochem, La Jolla, CA). Proteins in homogenates were analyzed using the bicinchoninic acid assay (BCA) method, and the samples were subsequently diluted with 2x Laemmli buffer (125 mM Tris-HCl pH 6.8, 4% w/v SDS, 10% 2-mercaptoethanol, 12% glycerol, and 0.004% w/v bromphenol blue) and heated in boiling water for 5 min. Aliquots of samples were loaded onto SDS-polyacrylamide gels. After separation on 4-12% gels, proteins were transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA) under 12V overnight, using the Bio-Rad Transblot apparatus (Hercules, CA). Membranes were blocked in 5% fat-free dry milk in TTBS (20 mM Tris/150 mM NaCl, pH 7.5, and 0.1% Tween-20) for 3 h and then incubated with one of the following primary antibodies overnight at 4 ^{o}C with gentle rocking: MTOR (Cell Signaling, 1:1000), phosphorylated MTOR (Cell Signaling, 1:1000), 4E-BP1 (Cell

Signaling, 1:1000), phosphorylated 4E-BP1 (Cell Signaling, 1:1000), p70S6 Kinase (Cell Signaling, 1:1000), or phosphorylated p70S6 Kinase (Cell Signaling, 1:1000). After being washed three times with TTBS, the membranes were incubated at room temperature (25 ^{o}C) for 2-3 h with a secondary antibody at 1: 50,000 (peroxidase-labeled donkey anti-goat or anti-rabbit IgG, Jackson Immuno Research). Finally, the membranes were washed with TTBS, followed by development using the Super Signal West Dura Extended Duration Substrate according to the manufacturer's instructions (Pierce, Rockford, IL). The signals were detected on Fujifilm LAS-3000 (Tokyo, Japan).

Results

Body weight gain of piglets

Table 4.2 summarizes the effects of oral administration of glycine to IUGR piglets on their growth performance. Based on body weight gain, the supplemental dose of 0.4 g glycine/kg BW yielded the most effective result. Glycine supplementation (0 to 0.4 g/kg BW) increased body weight in a dose-dependent manner. However, the growth rate of piglets receiving 0.8 g glycine/kg BW tended to be lower than that for the 0.4 g glycine/kg BW group. Based on body weight gain, the supplemental dose of 0.4 g glycine/kg BW yielded the most effective result. Glycine supplementation also had a beneficial effect on the growth of piglets with a normal birth weight (Table 4.3). Specifically, the average daily weight gain (ADG) in the first week and the second week was 155 and 149 g respectively, in the control group, compared to 210 g and 168 g, respectively, in the 0.4 g

glycine/kg BW group. As in IUGR piglets, the growth rate of normal birth weight piglets receiving 0.8 g /kg BW tended to be lower than that for the 0.4 g glycine/kg BW group.

Table 4.2. The effects of oral administration of glycine on the body weight of IUGR piglets (kg, n=10)

	Day 0	Day 7	Day 14
Control	0.86 ± 0.07	1.63 ± 0.08[a]	2.30 ± 0.12[a]
0.2 g Glycine / kg BW per day	0.88 ± 0.02	1.88 ± 0.06[b]	2.76 ± 0.08[b]
0.4 g Glycine / kg BW per day	0.85 ± 0.04	2.07 ± 0.05[b]	3.16 ± 0.14[c]
0.8 g Glycine / kg BW per day	0.88 ± 0.04	2.01 ± 0.08[b]	3.07 ± 0.07[c]

Different doses of glycine were administered orally to IUGR piglets between days 0 and 14 of life. Values are means ± SEM, n = 10 per group. Means in a column without a common superscript letter differ ($P < 0.05$) as analyzed by one-way ANOVA and the Student-Newman-Keuls (SNK) multiple comparison test.

Concentrations of amino acids in plasma

Glycine supplementation increased the concentrations of glycine in the plasma of neonatal piglets. Compared with the control group, supplementation with 0.2, 0.4, and 0.8g/kg glycine per kilogram body weight increased concentrations of glycine in plasma in a dose-dependent manner, by 1.52-, 1.94-, and 2.34- fold ($P < 0.05$), respectively (Figure 4.1). Concentrations of serine also increased in glycine treated piglets ($P < 0.05$). Concentrations of other amino acids in plasma (including arginine, aspartate, glutamate, histidine, hydroxyproline, lysine, ornithine, and proline) did not differ among the treatment groups of piglets.

Table 4.3. The effects of oral administration of glycine on the body weight of normal birth weight piglets (kg, n=10)

	Day 0	Day 7	Day 14
Control	1.30 ± 0.06	2.39 ± 0.07[a]	3.43 ± 0.12[a]
0.2 g Glycine / kg BW per day	1.32 ± 0.05	2.55 ± 0.11[ab]	3.62 ± 0.08[ab]
0.4 g Glycine / kg BW per day	1.29 ± 0.02	2.76 ± 0.05[b]	3.94 ± 0.07[c]
0.8 g Glycine / kg BW per day	1.30 ± 0.05	2.59 ± 0.08[ab]	3.72 ± 0.04[bc]

Different doses of glycine were administered orally to normal birth weight piglets between days 0 and 14 of life.

Values are means ± SEM, n = 10 per group. a-b Means in a column without a common superscript letter differ ($P < 0.05$) as analyzed by one-way ANOVA and the Student-Newman-Keuls (SNK) multiple comparison test.

Proteins in the MTOR cell signaling pathway in skeletal muscle

Western-blot analyses revealed that glycine supplementation induced the phosphorylation of MTOR pathway proteins, including MTOR, $p70^{S6K}$, and 4E-BP1, without altering expression of MTOR, $p70^{S6K}$, and 4E-BP1 proteins.

The expression of phosphorylated MTOR was 4.85 times greater in piglets receiving the 0.4 g glycine supplementation than for control piglets ($P < 0.05$, Figure 4.2). The 4E-BP1 and $p70^{S6K}$ proteins also had a high-level of phosphorylation in response to supplemental glycine ($P < 0.05$, Figures 4.3 and 4.4).

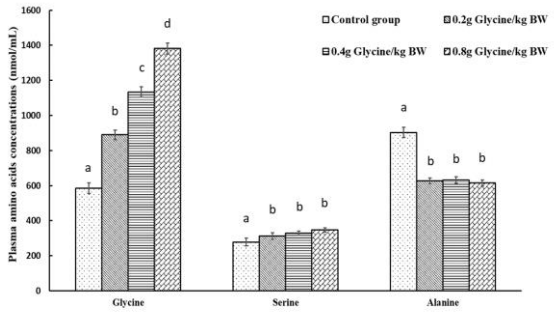


Figure 4.1. Plasma glycine, serine, and alanine concentrations in IUGR piglets.

Table 4.4. Plasma amino acid concentrations in IUGR piglets.

Amino acids (nmol/mL)	Control	0.2 g glycine/kg BW	0.4 g glycine/kg BW	0.8 g glycine/kg BW
Aspartic acid	14.1 ± 1.6	15.3 ± 1.7	14.7 ± 1.7	16.0 ± 2.5
Glutamate	189 ± 11.5^{a}	161 ± 10.9^{ab}	144 ± 8.91^{b}	137 ± 9.60^{b}
Asparagine	82.2 ± 6.1	83.4 ± 6.3	79.5 ± 9.9	75.2 ± 5.7
Serine	278 ± 12.5^{a}	312 ± 8.56^{b}	328 ± 11.7^{b}	347 ± 6.79^{b}
Glutamine	654 ± 18.4^{a}	547 ± 19.0^{b}	478 ± 20.5^{c}	455 ± 4.90^{c}
Histidine	67.2 ± 4.34	65.4 ± 3.37	64.6 ± 5.32	67.9 ± 7.18
Glycine	586 ± 10.3^{a}	890 ± 17.1^{b}	1134 ± 18.9^{c}	1382 ± 21.5^{d}
Threonine	154 ± 7.65^{a}	171 ± 6.11^{ab}	189 ± 11.4^{b}	194 ± 10.7^{b}
Citrulline	132 ± 4.09	136 ± 7.06	129 ± 7.72	134 ± 11.6
Arginine	172 ± 13.2	174 ± 10.3	181 ± 8.09	179 ± 12.7
β-Alanine	14.6 ± 2.17	14.1 ± 1.61	13.8 ± 1.03	14.4 ± 1.50
Taurine	124 ± 2.66	126 ± 10.72	129 ± 5.39	127 ± 3.80
Alanine	903±9.60^{a}	627±6.12^{b}	613±8.47^{b}	615±7.56^{b}
Tyrosine	154 ± 7.24	149 ± 4.71	156 ± 9.05	157 ± 8.23
Tryptophan	76.0 ± 5.14	73.2 ± 2.92	72.2 ± 5.61	76.1 ± 4.14
Methionine	60.1 ± 4.11	62.5 ± 5.27	67.5 ± 7.72	66.9 ± 4.93
Valine	210 ± 9.06	211 ± 5.09	216 ± 6.31	218 ± 4.20
Phenylalanine	71.5 ± 4.96	64.0 ± 3.90	71.0 ± 8.65	74.3 ± 5.77
Isoleucine	106 ± 5.81	112 ± 4.70	107 ± 9.43	110 ± 8.18
Leucine	151 ± 6.82	155 ± 3.97	153 ± 6.46	148 ± 8.41
Ornithine	114 ± 8.46	119 ± 6.25	115 ± 12.2	111 ± 6.30
Lysine	136 ± 11.4	141 ± 9.36	149 ± 10.6	153 ± 8.97

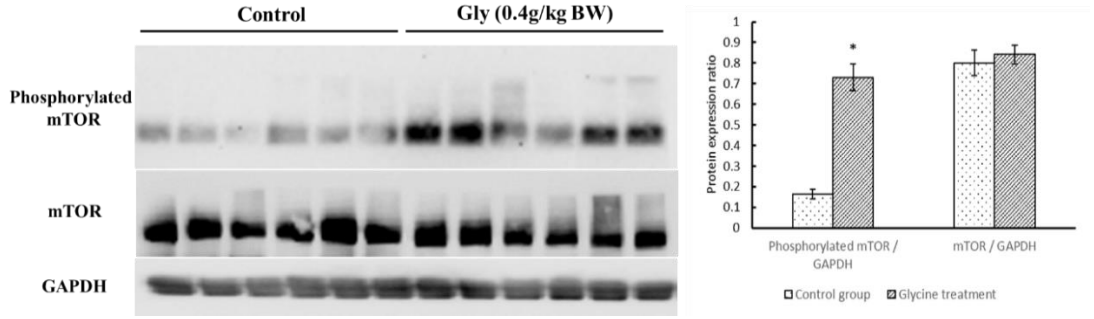


Figure 4.2. Abundances of MTOR and phosphorylated MTOR proteins in the skeletal muscle of IUGR piglet receiving oral administration of glycine. GAPDH protein was used as the internal control. Values are means ± SEM, n = 6 per group. * P < 0.05: different from the control group (P < 0.05).

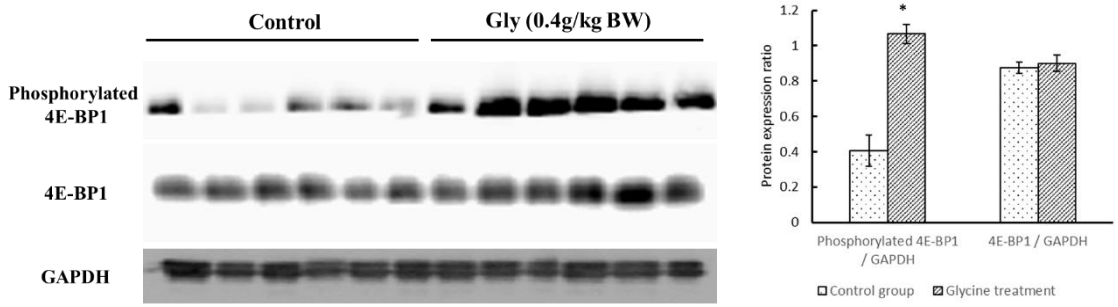


Figure 4.3. Abundances of 4E-BP1 and phosphorylated 4E-BP1 proteins in skeletal muscle of IUGR piglets receiving oral administration of glycine. GAPDH protein was used as the internal control. Values are means ± SEM, n = 6 per group. * P < 0.05: different from the control group (P < 0.05).

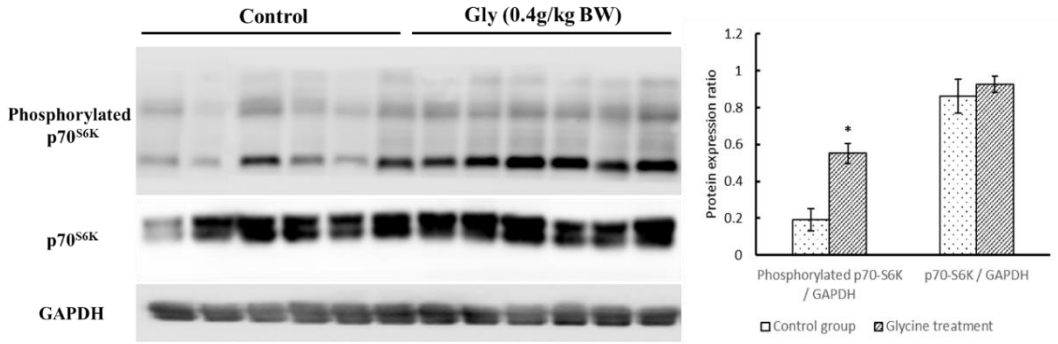


Figure 4.4. Abundances of p70S6K and phosphorylated p70S6K proteins in skeletal muscle of IUGR piglets receiving oral administration of glycine. GAPDH protein was used as the internal control. Values are expressed as means ± SEM, n = 6 per group. * P < 0.05: different from the control group (P < 0.05).

Discussion

Glycine has versatile roles in nutrition, metabolism, and health. It was classified as a "non-essential amino acid" for mammals, but this term has now been recognized as a misnomer (Hou and Wu 2017). Several lines of evidence have documented the beneficial effects of glycine on the maintenance and growth of mammals with a normal birth weight. First, glycine regulates the expression and distribution of mucosal barrier proteins (claudin-7 and ZO-3) in intestinal epithelial cells (Li et al. 2016). Second, glycine stimulates protein synthesis and inhibits proteolysis in skeletal muscle (Sun et al. 2016a), which is of enormous importance in both animal agriculture and human health (Hou et al. 2016). The underlying mechanisms involve the activation of the MTOR cell signaling (Ham et al. 2016; Koopman et al. 2017; Ost et al. 2015; Sun et al. 2016a) and reduced expression of pro-inflammatory cytokines (Liu et al. 2016). Third, dietary supplementation with glycine enhanced the growth performance and feed efficiency of milk-fed piglets (Wang et al. 2014a) and post-weaning piglets. To our knowledge, this is the first report regarding effects of glycine supplementation on the growth of sow-reared IUGR piglets.

Glycine is deficient in IUGR piglets, as its concentration in their plasma is only half that in normal piglets at days 1 and 7 of age. Insufficient endogenous synthesis of glycine (Chapter II) may contribute, in part, to growth restriction in IUGR neonates. A novel and important finding from this work is that oral administration of glycine to normal and IUGR piglets enhanced their growth, with the dose of 0.4 g glycine/kg BW being the most effective. Interestingly, in contrast to supplementation with leucine (Sun et al. 2015) or

protein (Jamin et al. 2010), both normal and IUGR piglets responded similarly to oral administration of glycine with an improvement of lean tissue gain. It is possible that most of the supplemental glycine is used for whole-body protein synthesis and, therefore, only a small amount of glycine is oxidized to generate ammonia in all of the neonates. Hence, no toxicity occurs in association with glycine supplementation. In this regard, glycine offers an advantage over leucine or protein as a nutritional supplement to IUGR neonates, although both amino acids can activate MTOR cell signaling and protein synthesis in skeletal muscle. Our results also provide another line of evidence to support the notion that endogenous synthesis of glycine is insufficient for maximal growth and feed efficiency in young pigs.

The MTOR signaling pathway is the master regulator of protein biosynthesis, as well as cell growth and cytoskeleton remodeling (Dennis et al. 2011; Wu 2013b). Skeletal muscle development is highly dependent on MTOR activation. The MTOR system consists of two components: MTOR complex 1 (MTORC1) and MTOR complex 2 (MTORC2). Both of MTORC1 and MTORC2 can be activated by an upstream protein kinase (Kim and Guan 2011; Sun et al. 2016a). Activated MTOR phosphorylates two downstream target proteins: S6K1 and 4E-BP1. The cell signaling cascade initiated by MTOR phosphorylation leads to protein synthesis in tissues (including skeletal muscle) (Dennis et al. 2011; Kim and Guan 2011). Results of the present study indicated that glycine supplementation to neonatal IUGR piglets enhanced the phosphorylation of MTOR, $p70^{S6K}$, and 4E-BP1 proteins in skeletal muscle, as reported for muscle cells (Sun et al. 2016; Liu et al. (2016). Glycine is virtually not degraded by muscle cells (Wu 2013).

Therefore, it is unlikely that a metabolite of glycine mediates its effect on MTOR activation. We propose that glycine binds to MTOR and alters the configuration of the protein kinase. This would be analogous to the binding of glycine to the glycine receptor in the central nervous system, thereby modulating neurotransmission (Yevenes and Zeilhofer 2011). Thus, the possibility that glycine is an allosteric activator of MTOR should be examined in future studies.

In conclusion, oral administration of glycine (0.2, 0.4 and 0.8 g/kg BW per day) enhanced growth performance in both normal birth weight and IUGR piglets. Glycine activated the MTOR cell signaling pathway to promote the net deposition of protein in skeletal muscle and the whole body. At the supplemental doses, all piglets tolerated glycine with no side effects. These results indicate a functional role for glycine in metabolism and support the view that endogenous synthesis of glycine in young pigs cannot meet their metabolic needs for maximal growth.

CHAPTER V

SUMMARY AND DIRECTION OF FUTURE RESEARCH

Glycine is one of the most abundant amino acids in animals and it plays vital roles in nutrition, metabolism, reproduction, and health. However, sow's milk provides at most less than 30% of the metabolic needs for glycine by suckling piglets. Therefore, endogenous synthesis of glycine must exist in young pigs to support their growth and development. Serine, threonine, and choline are substrates for glycine synthesis in the liver, but these pathways contribute only a small amount of glycine to sow-reared piglets due to the limited provision of the glycine precursors from milk. New endogenous source(s) of glycine in pigs remain to be identified. A series of experiments were conducted to fill in this gap of knowledge.

The major purpose of this dissertation research was to investigate the novel endogenous glycine synthesis pathway. A substantial body of literature documented a new function for dietary glycine in improving growth performance of young pigs and ameliorating cell injury (Wang et al. 2013a; Wu et al. 2014). For example, dietary glycine supplementation to sow-reared piglets is necessary for them to realize their maximal growth potential (Powell et al. 2011; Wang et al. 2014c). Results of this dissertation research indicate that the amount of endogenously synthesized glycine does not meet the metabolic requirements by sow-reared piglets.

The first part of this study was designed to investigate a role of hydroxyproline (an abundant amino acid in sow's milk and plasma of piglets) in glycine synthesis by tissues of newborn piglets (Chapter II). Neonatal piglets at 0, 7, 14 and 21 days of age were used. The enzymatic activities, as well as expression of mRNAs and abundances of the hydroxyproline-glycine pathway proteins, including OH-POX, POX, AGT, and HOA, were present in the liver and kidneys at birth and during the suckling period. These enzymes were expressed at lower levels in the pancreas, small intestine, and skeletal muscle of 14- to 21-day-old pigs. Based on the mass of tissues, we conclude that the liver (mainly periportal hepatocytes) and kidneys play an important role in glycine synthesis in piglets during the first days of their postnatal life. As pigs grew toward weaning, hepatic OH-POX was expressed mainly in the perivenous hepatocytes (a small population of hepatocytes), the small intestine and the skeletal muscle became the major sites of glycine synthesis from hydroxyproline. The coordination of multiple organs maximizes net glycine production by piglets.

The concentration of glycine in the plasma of IUGR piglets was much lower than that in normal birth weight piglets. This raised a question of whether glycine synthesis was reduced in IUGR piglets. Thus, the second part of the research investigated glycine synthesis from hydroxyproline by tissues of IUGR piglets (Chapter III). The activities of OH-POX and SHMT were lower in the liver, kidneys and other tissues of IUGR piglets, compared with normal birth weight pigs. The activity of TDH did not differ between IUGR and normal piglets. Similar results were obtained for expression of mRNAs for those enzymes in a tissue-specific manner. The capacity for the conversion of hydroxyproline

into glycine in the tissues of IUGR piglets was only about 10% of that for normal piglets. Collectively, these novel results provide the first line of evidence that endogenous synthesis of glycine is impaired sow-reared pigs piglets.

Subsequent experiments were conducted to evaluate the role of endogenous synthesis of glycine in growth of piglets (Chapter IV). Particularly, glycine supplementation was used as a tool to assess whether glycine synthesis may limit lean tissue gain in both IUGR and normal piglets. Both types of piglets responded well to oral administration of 0.4 g glycine/kg BW with improved growth performance. This is in contrast to the reported adverse effects of leucine or protein supplementation on IUGR piglets, which include high rates of morbidity and mortality possibly due to ammonia toxicity. It is likely that most of the supplemental glycine is used for protein synthesis rather than oxidation to ammonia and CO_2. Consistent with its growth-promoting effect, glycine supplementation enhanced the phosphorylation of mechanistic target of rapamycin (MTOR), eukaryotic initiation factor 4E binding protein 1 (4E-BP1) and p70S6 kinase (p70S6K) in skeletal muscle, leading to an increase in net protein synthesis by the tissue. Collectively, these findings indicate that glycine is a signaling molecule that has vital roles in young pigs and that the endogenous synthesis of glycine is insufficient for maximal growth in the neonatal pig. Future studies are needed to better understand the metabolic zonation of glycine-synthetic enzymes in the liver and the molecular mechanisms responsible for differential expression of OH-POX in hepatocytes with age. It will also be of interest to investigate relationships between low rates of glycine synthesis (low levels of enzyme expression) and malnutrition in IUGR piglets. Finally, a role for glycine in placental growth, development, and

angiogenesis warrants studies, with the hope of preventing intrauterine growth restriction (IUGR) in pigs. Finally, because glycine is involved in one-carbon unit metabolism and, therefore, methylation of genes and proteins, it is imperative to determine whether supplementing glycine to gestating swine or neonatal pigs may affect epigenetic modifications and have carry-on effects to improve growth performance and feed efficiency of offspring. Collectively, the novel findings from this dissertation provide a biochemical basis for the classification of glycine as a nutritionally essential amino acid for young pigs and revision of the century-old nutritional concept that dietary glycine is not needed by mammals. Findings from the current study not only advance basic knowledge of nutritional biochemistry, but also have important implications for developing practical means to enhance pig production in the animal industry. Finally, because the pig is an excellent model for studying human nutrition and metabolism, results from this dissertation research may be used to formulate new diets for IUGR human infants.

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APPENDIX

A1. Preparation of Tissue Homogenates for Enzyme Assays

Chemicals.

1. 5 mM Hepes (pH 7.4): Dissolve 596 mg Hepes (Sigma, Cat. #H-3375) in 480 ml H_2O. Adjust to pH 7.4 with 10 M NaOH. Makeup to final volume 500 ml with H_2O.
2. 300 mM Sucrose/1 mM EDTA/5 mM Hepes (pH 7.4): Dissolve 51.4 g sucrose and 186 mg EDTA (disodium) in 500 ml of 5 mM Hepes buffer (pH 7.4).
3. A stock solution of protease inhibitors (2.5 mg/ml each): Dissolve 2.5 mg aprotinin, 2.5 mg chymostatin, 2.5 mg pepstatin A, and 2.5 mg phenylmethylsulfonylflouride in 1 ml of dimethyl sulfoxide.
3. Homogenization buffer (300 mM sucrose/1 mM EDTA/5 mM Hepes/3 mM DTT/0.5% Triton X-100, pH 7.4): Mix 30 ml of 300 mM sucrose/1 mM EDTA/5 mM Hepes solution (pH 7.4), 30 μl stock solution of protease inhibitors, 15 mg dithiothreitol, and 0.15 ml Triton X-100.

Tissue homogenization:

1. Rinse a fresh tissue twice with saline (0.9% NaCl) to remove blood.
2. Obtain a sample of ~0.5 g.
3. Homogenize the sample (~0.5 g) in 2 ml of homogenization buffer.
4. Transfer the 2-ml homogenates to a tube.
5. Rinse the homogenizer with 3 ml of the homogenization buffer, and transfer it to the tube containing the previous 2-ml homogenates.
6. The combined homogenates are centrifuged at 3000 g, 4^{o}C for 5 min, and the supernatant fluid is used for enzyme assay. This supernatant fluid is stored at -80^{o}C.

A2. Determination of 4-Hydroxy-2-Oxoglutarate Aldolase Activity

Chemicals.

1. Buffer: 100 mM potassium phosphate (KPi) buffer (pH 7.8)
2. Substance: 100 mM DL-4-hydroxy-2-ketoglutarate (disodium salt)

Assay procedures

1. To each tube, add the following:

Sample tube	Blank tube
0.6 mL buffer	0.6 mL buffer
0.2 mL Tissue homogenate	0.2 mL Tissue homogenate
0.2 mL H_2O	0.2 mL H_2O
Warm up to 37^oC	
0.2 mL 100 mM H.O.A	0.2 mL H_2O

2. After 0.5, 5.5, and 10.5 min, remove 0.3 mL from system into 0.3 mL 10% TCA
3. Centrifuge 2500 rpm for 10 min, acquire the supernatant
4. Measure the pyruvate production by following pyruvate analysis protocol

A3. Determination of Alanine: Glyoxylate Transaminase Activity

Chemicals.

1. Buffer: 100 mM KPi buffer (pH 7.4)
2. Substance: 100 mM glyoxylic acid
   Alanine: 100 mM

Assay procedures

1. To each tube, add the following:

Sample tube	Blank tube
0.25 mL buffer	0.25 mL buffer
0.1 mL Glyoxylic acid	0.1 mL H_2O
0.1 mL H_2O	0.1 mL H_2O
Warm up to 37^oC	
0.02 mL Tissue homogenate	0.02 mL Tissue homogenate

2. After 20 min incubation, stop the reaction by using 0.5 mL 10% TCA
3. Centrifuge 2500 rpm for 10 min, and acquire the supernatant
4. Measure pyruvate production by following pyruvate analysis protocol

A4. Determination of Hydroxyproline Oxidase Activity

Chemicals.

1. Buffer: 80 mM KPi (pH 8.0) with 16 uM Cytochrome:
   Dilute 4 mg Type III Cytochrome C into 20 mL buffer
2. Substance: 600 mM 4-hydroxy-L-Proline. Dissolve 0.787 g 4-hydroxy-L-proline into 10 mL H_2O
3. Reaction reagent: Dissolve 40 mg *O*-aminobenaldehyde into 16 mL 100% ethanol thoroughly, and combine the ethanol with 24 mL 10% TCA

Assay procedures

1. To each tube, add the following:

System:	Sample tube	Blank tube
	1.5 mL Buffer	1.5 mL Buffer
	0.75 mL Hydroxyproline	0.75 mL H_2O
	0.45 mL H_2O	0.45 mL H_2O
	Warm up to 37^oC	
	0.3 mL Tissue homogenate	0.3 mL Tissue homogenate

2. After 0, 15, and 30 min, remove 0.8 mL from the assay solution into 1.6 mL reaction reagent
3. Leave the tube for at least 30 min at room temperature, and centrifuge the tube at 2500 rpm for 10 min. Acquire the supernatant fluid.
4. Measure the absorbance of the supernatant fluid at 443 nm.

Calculation:

$$\frac{(\Delta A_{443} \text{ / min for sample} - \Delta A_{443} \text{ / min for blank}) \times 3.75 \times 1000}{2.59 \times \text{Tissue weight (g)} \times \text{Reaction time (min)}}$$

A5. Determination of Proline Oxidase Activity

Chemicals

1. 50 mM Potassium Phosphate Buffer (pH 7.2):
   a) 50 mM K_2HPO_4: Dissolve 8.7 g of K_2HPO_4 in 1 L of deionized H_2O.
   b) 50 mM KH_2PO_4: Dissolve 6.8 g of KH_2PO_4 in 1 L of deionized H_2O.
   c) 50 mM Potassium Phosphate Buffer (pH 7.2):
   d) 50 mM Potassium Phosphate Buffer (pH 7.5):
2. 30 mM Proline: Dissolve 35 mg proline in 10 ml of 50 mM potassium phosphate buffer (pH 7.5).
3. 200 µM Ferricytochrome C: Dissolve 2.5 mg ferricytochrome C (horse heart) in 1 ml of 50 mM potassium phosphate buffer (pH 7.5).
4. 100 mM o-aminobenzaldehyde: Dissolve 24.2 mg o-aminobenzaldehyde in 0.842 ml of 95% ethanol. Add 1.158 ml H_2O, and mix.

Assay procedures

1. To each tube, add the following:

   0.5 ml of 30 mM proline 0.1 ml of 200 µM ferricytochrome C

   0.1 ml of sonicated mitochondria suspension

   0.3 ml of 50 mM potassium phosphate buffer (pH 7.5)

   (Blanks: All above components, but 0.5 ml of 10% TCA is added to the assay mixture before addition of 0.1 ml mitochondrial suspension.)

2. Incubate the assay mixture at 37^{o}c for 30 min.

3. Add 0.5 ml of 10% TCA to terminate the reaction. (Note that blanks are already acidified with 10% TCA before addition of cell extracts.)

4. Add 0.1 ml of 100 mM *o*-aminobenzaldehyde to the acidified solution. Mix.

5. After 30 min, centrifuge the solution at 600 g for 5 min. Measure the absorbance of the supernatant fluid at 440 nm.

Calculation:

Molar extinction coefficient of POX at 440 nm = $2.7x10^3$/M.cm

Total volume of the solution: 1.6 ml.

$$\frac{(\Delta A_{440} / \text{min for sample} - \Delta A_{440} / \text{min for blank}) \times 1.60 \times 1000}{2.70 \times \text{Tissue weight (g)} \times \text{Reaction time (min)}}$$

A6 Pyruvate Assay Using Fluorometric Method

Chemicals.

1. 150 mM Phosphate buffer (pH 7.5): Dissolve 10.2 g KH_2PO_4 in 450 ml H_2O. Adjust the solution to pH 7.5 with 10 M KOH, and makeup to a final volume of 500 ml with H_2O.
2. β-NADH solution:

   a) 1 mM NADH: Dissolve 7.1 mg ß-NADH-Na_2 (Boehringer Mannheim, Cat # 128-023) in 10 ml of 150 mM phosphate buffer (pH 7.5).

   b) 50 μM NADH: Dilute 0.5 ml of 1 mM NADH to 10 ml with 150 mM buffer.
3. Pyruvate standard:

   a) 5 mM Pyruvate: Dissolve 55 mg sodium pyruvate in 100 ml H_2O.

   b) 50 μM Pyruvate: Dilute 0.1 ml of 5 mM pyruvate to 10 ml with H_2O.

Pyruvate STD (nmol/ml)	H_2O (ml)	50 μM Pyruvate
0	1.0	0.0
5	0.9	0.1
10	0.8	0.2
15	0.7	0.3
20	0.6	0.4
25	0.5	0.5

4. L-Lactate dehydrogenase from beef heart.

Assay Procedures.

1. To each tube, add the following:

   0.2 ml of Pyruvate standard/Sample

   0.15 ml of 50 μM NADH

   1.5 ml of 150 mM Phosphate buffer (pH 7.5)
2. Mix well. After 2 min, read fluorescence F_1 (Excitation 340 nm, Emission 460 nm).
3. Add 5 μl L-lactate dehydrogenase into each tube. Mix well. After 5 min, read fluorescence F_2 (Excitation 340 nm, Emission 460 nm).